

**STRUCTURE, FUNCTION AND PLASTICITY OF
CONVERGENT SYNAPSES
IN
REINNERVATED MUSCLE**

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DECLARATION

I declare that the work described in this thesis and its composition are entirely my own.

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ABSTRACT

Adult mammalian neuromuscular junctions (NMJs) are characterised by a highly stereotyped pattern of innervation: each muscle fibre is contacted at the motor endplate region by the nerve terminal boutons belonging to a single axon. However, during development, and at adult NMJs after nerve injury, the process of synapse elimination brings about the transition from innervation by multiple motor axons to innervation by only one. Although the precise mechanisms governing the passage from polyneuronal to mononeuronal innervation remain unclear, differences in the timing and amount of neuromuscular activity are currently believed to be responsible. However, there is evidence that activity alone merely influences rather than determines the outcome of synapse elimination. First, adult rat fourth deep lumbrical (4DL) muscles can retain polyneuronal innervation indefinitely, following reinnervation and recovery from a period of paralysis; second, synapse elimination may proceed in these muscles in the absence of evoked activity.

The experiments described in this thesis were directed towards clarifying the role of activity in sculpting patterns of innervation at adult reinnervated rat NMJs. Three hypotheses were tested: first, that activity is necessary for synapse elimination; second, that activity is sufficient for synapse elimination; and third, that activity shapes neuromuscular connections during critical periods of plasticity.

To test the first hypothesis, 4DL muscles were subjected to partial denervation and TTX paralysis, but were not allowed to recover from the activity block. Vital

staining and immunocytochemistry revealed that competitive synapse elimination had taken place at some endplates, even when evoked and spontaneous activity was abolished throughout the muscles.

To test the second hypothesis, 4DL muscles were subjected to partial denervation and TTX paralysis, but were allowed to recover for at least two weeks from the paralysis. Polyneuronally innervated 4DL NMJs were examined to ask whether terminals contributing relatively small fractional occupancies of the endplate (<49% of total endplate area) were disproportionately weak and, in fact, in the process of being eliminated from endplates. Polyneuronally innervated NMJs were identified with the vital dyes FM1-43 and RH414, which both stain recycling synaptic vesicles. Simultaneous intracellular recording and synaptic imaging were used to directly correlate input strength with size. Results showed that when small inputs were normalised for size, they were just as efficacious as larger converging inputs to polyneuronally innervated endplates.

Finally, to test the third hypothesis, the same paradigm as that described immediately above was used, but animals were sacrificed 0, 7, and 14 days after the resumption of activity from TTX paralysis to ask how structure: function relationships change during a known period of synapse elimination. Intracellular recording and FM1-43/RH414 were used to identify the morphological and physiological correlates of synapse elimination. Results from these experiments indicated that synapse elimination progresses slowly between days 1-7, and rapidly between days 7-14, but 4DL muscles did not return to a state of mononeuronal innervation two weeks

after the resumption of activity. These results suggest that activity has a limited window of opportunity in which to effect the outcome of neuromuscular synaptic plasticity.

Taken together, these results show that activity, though influential, is neither necessary nor sufficient for the competitive elimination of synapses in reinnervated muscle. Further, the data suggest that the timing of transmitter-receptor activation is not the only mechanism by which inputs are removed during neuromuscular synapse elimination. Alternative mechanisms that may be operating during the competitive reinnervation of skeletal muscle are discussed.

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ABBREVIATIONS

α -BTX	Alpha-bungarotoxin
4DL	Fourth deep lumbrical (muscle)
Ach	Acetylcholine
AchR	Acetylcholine receptors
AchE	Acetylcholinesterase
BDNF	Brain-derived neurotrophic factor
BoTox	Botulinum toxin
CNTF	Ciliary neurotrophic factor
EM	Electron microscopy
EMG	Electromyogram
E:O index	Efficacy:occupancy index
EPP	Endplate potential
FITC	Fluorescein Isothiocyanate
GDNF	Glial cell line-derived neurotrophic factor
LGN	Lateral geniculate nucleus
LPN	Lateral plantar nerve
LTP	Long term potentiation
MEPP	Miniature endplate potential
N-CAM	Neural cell adhesion molecule
NF	Neurofilament
NGF	Nerve growth factor
NT	Neurotrophin
PBS	Phosphate buffered saline
QC	Quantal content
QC:O index	Quantal content:occupancy index
SC	Superior Colliculus
SD	Sprague Dawley
SN	Sural Nerve
tfl	Tensor fasciae latae (muscle)
TRITC- α -BTX	Tetramethylrhodamine isothiocyanate-conjugated alpha bungarotoxin
TTX	Tetrodotoxin

Chapter 1: Introduction

1.1 Background

The nervous system is an elaborate assembly of cells that continually receives and processes information, and executes complex decisions. In order to fulfil the task of determining many aspects of behaviour, the basic units of the nervous system – neurons – each make and receive hundreds or thousands of synaptic connections. In general, the changes in synaptic connectivity and function that neurons are capable of undergoing in response to experience may form the cellular substrates of more complex cognitive and behavioural functions. Thus, an understanding of the factors that determine the structure and function of individual synapses is essential to an understanding of how the nervous system allows us to learn and remember, produce complex movements, and controls consciousness and perception.

From a variety of studies over the past 30-35 years, it has become apparent that substantial alterations of synaptic connections occur in the developing nervous system. For example, neurons in many regions are overproduced initially and compete for survival in early embryonic life (reviewed by Purves & Lichtman, 1980). Another developmental adjustment is the elimination of some of the synaptic connections initially formed between pre- and postsynaptic targets. Since the hallmark of this synaptic remodelling is a reduction in the amount of neuronal convergence and divergence, it has come to be called “synapse elimination”. Although synapse elimination has been described in a range of structures and pathways throughout the nervous system, the process has been particularly well documented at neuromuscular

synapses (recently reviewed by Ribchester, 1999, in press; Sanes & Lichtman, 1999). In contrast to many central synapses, the neuromuscular junction is structurally simple and easily accessible to experimental manipulation and observation. For these reasons, the neuromuscular junction has been exploited as a model system in which to study the processes governing synapse elimination – processes which may be common to the plasticity that occurs throughout the nervous system.

One way the neuromuscular junction has been exploited in the study of synapse elimination is in evaluating the role of activity. Interest in the question of how experience shapes neural connections is a general one in neuroscience. The neuromuscular junction is a useful system in which to make attempts to answer this question because it presents sets of synapses which are undergoing remodelling during a time in neonatal life when organisms are just beginning to use their muscles for voluntary, co-ordinated movement. The role of use and disuse of synaptic connections during this time seems to play an important part in determining their maintenance or elimination.

1.2 Purpose and Overview of the Present Study

The experiments described in this thesis were directed toward an understanding of the role that activity plays in neuromuscular synapse elimination. In the following chapters, new evidence suggesting that activity is influential, but not decisive in sculpting patterns of innervation at the neuromuscular junction is presented. In the remainder of

this chapter, a description of the neuromuscular junction, its formation, function, and modes of plasticity will be given. The materials and methods used for the investigation will be given in Chapter 2. In Chapter 3, the results of a test of the necessity of activity for synapse elimination will be presented. Chapter 4 comprises experiments on the sufficiency of activity to effect synapse elimination. Chapter 5 presents data concerning the structural and functional correlates of synapse elimination, in order to investigate the possibility that there are critical periods for neuromuscular plasticity. Finally, Chapter 6 presents a general discussion of the results described in the preceding chapters in light of current and previous findings.

1.3 The Neuromuscular Junction

The neuromuscular junction is the chemical synapse formed between spinal motor neurons, arising in the central nervous system, and their targets in the peripheral nervous system, the skeletal muscle fibres (Purves & Lichtman, 1985). Like other chemical synapses, the neuromuscular junction consists of two primary elements: the presynaptic nerve terminal and the postsynaptic apparatus, formed at a discrete region on the postsynaptic membrane, also known as the motor endplate.

1.3.1 A Brief History

The structure of the neuromuscular junction was the focus of experimental investigations as early as the 1800's, when Kuhne (1888) used gold chloride staining techniques to describe the motor nerves that innervate skeletal muscle. Later, Tello, a

student of Cajal, used silver staining methods and the light microscope to elucidate some of the main features of the neuromuscular junction, including the presence of polyneuronal innervation (in Cajal, 1928). The presence of polyneuronal innervation in neonatal muscles was later confirmed by Boeke (in Cajal, 1928).

An understanding of neuromuscular transmission has really only been clear since (approximately) the 1950's. For nearly 1500 years, Galen's gaseous "animal spirits", thought to be produced in the base of the brain and carried throughout the body in hollow nerves, were believed to cause muscle contraction (see Hubbard et al., 1969). The idea of nerve-muscle transmission was advanced by Pollitzer (1886), on the basis of an earlier report that an arrow poison (curare) used by some South American Indian tribes abolished nerve-evoked muscle contraction. Entering what he called "the region of pure speculation", Pollitzer suggested that curare acted on the nerve terminal endings "in such a way that the nervous impulse can no longer pass through". In 1921, Otto Loewi published the results of a series of experiments that demonstrated unambiguously that nervous system activity could be transmitted from one region to another by the release of a chemical messenger. Loewi stimulated the vagus nerve of a frog heart containing a small volume of physiological saline in the ventricles. He observed a decrease in heart activity, and then transferred fluid from the stimulated heart into a test heart that lacked vagal innervation. This test heart also exhibited a decrease in activity, indicating that a substance Loewi called "Vagusstoff" had transmitted the effect of stimulation to the test heart. By the 1930's, it had been established that muscle

contraction was in fact due to the release of the neurotransmitter acetylcholine (Ach) from the nerve terminals (Dale & Dudley, 1929; Dale & Feldberg, 1934; Dale et al., 1936).

In the 1950's, the then-new techniques of intracellular recording (Ling & Gerard, 1949; Fatt & Katz, 1951; Hodgkin & Huxley, 1952) and electron microscopy (Palay & Palade, 1955; DeRobertis & Bennett, 1955) were applied to the adult neuromuscular junction, resulting in a more complete view of its structure and function. These studies led to a detailed investigation of the development and plasticity of this synapse that culminated in the 1970's with the rediscovery of Tello's original observations on polyneuronal innervation (Redfern, 1970; see below, section 1.5 for a detailed description). Since then, technical advances in imaging and molecular biology have permitted detailed investigations of the way in which the structure and function of neuromuscular synapses develop and change throughout the life of the organism.

1.3.2 Presynaptic Structure

The nerve terminal contains large numbers of 50nm diameter synaptic vesicles (Hirokawa et al., 1989), the existence of which was first established by electron microscopy (EM; DeRobertis & Bennett, 1955). Soon after this discovery, synaptic vesicles were isolated and shown to contain the neurotransmitter acetylcholine (Ach; Whittaker et al., 1964). Within the nerve terminal boutons, synaptic vesicles are clustered at specialised release sites known as active zones (Hirokawa et al., 1989). At

the active zones is found much of the molecular machinery that regulates vesicle docking, fusion, and exocytosis, such as calcium channels (Robitaille et al., 1990) calcium-gated potassium channels (Robitaille et al., 1993), neurexins (Ushkaryov et al., 1992) and syntaxin (Bennett et al., 1992).

1.3.3 Postsynaptic Structure

The postsynaptic apparatus formed by the muscle fibre is characterised by invaginations of the membrane called junctional folds and a high concentration of acetylcholine receptors (AChRs) packed in the membrane at the tops of the folds (see Salpeter, 1987). Other membrane proteins, including neural cell adhesion molecule (N-CAM; Covault & Sanes, 1986), voltage-gated sodium channels (Flucher & Daniels, 1989; Boudier et al., 1992), and cytoskeletal proteins such as a 43kD acetylcholine receptor associated protein (Neubig et al., 1979; Porter & Froehner, 1983) are also concentrated in the postsynaptic apparatus of the motor endplate.

1.3.4 Extrasynaptic Structure

Although precisely aligned with one another, nerve terminals do not directly contact muscle fibres, but are separated by a 50nm synaptic cleft (Robertson, 1956). Running through the synaptic cleft and extending down into the junctional folds is an extracellular matrix called the basal lamina. The basal lamina forms a sheath approximately 23-30nm thick around each muscle fibre and myelinated axon, and at the motor endplate extends into the synaptic cleft, following the contours of the junctional

folds (Hirokawa & Heuser, 1982). The synaptic basal lamina is biochemically distinct from that which surrounds the remainder of the axons and muscle fibres. At the motor endplate, the basal lamina contains a high concentration of acetylcholinesterase (AChE; Hall, 1973), and specific isoforms of other extracellular matrix molecules, including agrin (Reist et al., 1987), laminin (Hunter et al., 1989), and entactin (Chiu & Ko, 1994).

1.3.5 Schwann Cells

Schwann cells in the peripheral nervous system exist in two forms: myelin-forming and non-myelin-forming, both of which arise from a common pool of immature Schwann cells during development (Rickman et al., 1985; Loring & Erickson, 1987). The difference between the two Schwann cell types is reflected in their molecular makeup: only the myelin-forming cells express a series of myelin proteins, including P₀, myelin basic protein, myelin-associated glycoprotein and P₂ (Hahn et al., 1987; Martini & Schachner, 1986). The non-myelin-forming Schwann cells express another set of proteins, including NGF receptors, the intermediate filament protein GFAP and N-CAM (Jessen & Mirsky, 1984; Noble et al., 1985; Mirsky et al., 1986), suggesting that their function is to insulate nerve terminals from the local environment.

1.3.6 The Physiology of Neuromuscular Transmission

The generally accepted view of the synthesis and release of ACh involves a complex cycle of events. ACh is first formed in the cytoplasm of the nerve terminal from acetyl CoA and choline, in a reaction catalysed by the enzyme choline acetyltransferase

(Tucek, 1984). It is then accumulated by an Ach "transporter" to a high concentration within the vesicles. Release of Ach, a strongly calcium-dependent process (del Castillo & Stark, 1952), involves fusion of the vesicles with the synaptic membrane of the nerve terminal, resulting in the release of a "quantum" of several thousand Ach molecules into the synaptic cleft (Fatt & Katz, 1952; Kuffler & Yoshikami, 1975)¹. Some of these molecules bind to the AchRs while the rest are rapidly broken down by AchE in the basal lamina. The choline thus formed is taken back up into the terminal by a high-affinity uptake system, making it available for the re-synthesis of Ach (Tucek, 1984).

The release of Ach normally gives rise, after a delay, to a large depolarising potential in the postsynaptic membrane, which usually reaches threshold and produces a muscle fibre action potential. The process underlying the action of Ach on the muscle fibre is one in which a molecule (or several molecules) of Ach combine(s) with an AchR, which then undergoes a conformational change resulting in the opening of cationic channels (most importantly, a sodium/potassium channel; Fatt & Katz, 1951; Takeuchi & Takeuchi, 1960; Jenkinson & Nicholls, 1961). The dependence of sodium and potassium permeabilities on the membrane potential is responsible for the action potential: the magnitude and timing of the permeability changes to the two ions accounts for the rising and falling phases (Fatt & Katz, 1951; Magleby & Stevens, 1972).

¹ A quantum simply describes the smallest unit in which the transmitter is normally secreted. In general, the number of quanta released by depolarisation (the *quantal content* of a synaptic response) may vary, but the number of molecules in a quantum (the *quantal size*) is relatively fixed (Fatt & Katz, 1952; but see Dennis & Miledi, 1974; Martin, 1977).

Quantal Release

One of the earliest observations resulting from intracellular recording from muscle fibres at neuromuscular junctions was the presence of spontaneous miniature endplate potentials (MEPPs), resulting from the apparent synchronous release of many molecules of Ach from the terminal boutons (Fatt & Katz, 1952). The time course of a MEPP resembled that of the endplate potential (EPP) resulting from nerve stimulation. When the amplitude of the EPP was reduced by lowering external calcium (and raising magnesium), the EPP showed striking variability, with the smallest EPPs comparable in amplitude to MEPPs. These observations led to the quantal hypothesis of transmitter release: an EPP results from the release of a number of packets of Ach from the nerve terminal, each packet containing the amount of Ach that would by itself produce a MEPP (Fatt & Katz, 1952).

1.4 Neuromuscular Development and Synapse Formation

The formation of synapses requires a series of steps, including the generation of neurons and their target cells, the guidance of axons to their targets, and the induction of a specialised presynaptic terminal and postsynaptic membrane.

1.4.1 Muscle Genesis

During development, the first indication of segmentation in the embryo is provided by the somites, which are a component of the neural crest (Purves & Lichtman, 1985). The

somites ultimately give rise to a number of structures, including the vertebrae, ribs, dermis and skeletal muscle fibres. The first step in the generation of the peripheral nervous system is when mesodermal cells within the somites acquire a myogenic identity and migrate to sites where muscles eventually form. In mammals, a family of myogenic regulatory factors are involved in conferring a muscle phenotype onto mesodermal cells during development. The initial member of the family to be discovered was MyoD, which was identified by its ability to convert cultured fibroblasts into skeletal myoblasts (Davis et al., 1987). The other members of this family, in vertebrates, are Myf-5, myogenin and MRF4 (reviewed by Grinnell, 1995). Each of these factors has the potential to turn tissue culture cells into myoblasts, which in turn fuse with one another and differentiate into muscle.

1.4.2 Motor Neuron Genesis

Motor neurons arise in the brainstem and ventral horn of the spinal cord from multipotential progenitor cells (Leber et al., 1990). Axons exit the spinal cord through ventral roots, and travel to innervate given muscles; several axons innervating the same muscle exit the ventral root together and join to form a spinal nerve. Motor axons do not branch *en route* from spinal cord to target muscle. Thus, in mammals, each skeletal muscle fibre is normally innervated by a single motor neuron; however, that motor neuron may branch at the level of the muscle to innervate many fibres. The combination of one motor axon/neuron and all the muscle fibres it innervates is called a motor unit.

1.4.3 Schwann Cell Genesis

Like the muscle fibres and motor axons, Schwann cells arise from cells in the neural tube (Rickman et al., 1985; Loring & Erickson, 1987). Although the exact location that Schwann cells become associated with motor axons is unclear, it is likely that the two cell types meet within or near the somites, before travelling through the periphery to the muscles.

Current ideas about the ways motor axons and Schwann cells travel to and find target muscles suggest that the motor axons decipher guidance signals, and Schwann cells follow (Riethmacher et al., 1997). The navigating signals that these cells respond to are not known, but several candidate molecules have been suggested to act alone and in concert to ensure accurate guidance. For example, the extracellular matrix molecule laminin-1 has been shown to stimulate the growth and extension of axons (Lander, 1989); the cell surface molecule cadherin promotes cell adhesion (Takeichi, 1995); and the secreted factor netrin-1 mediates the repulsion of axons from incorrect pathways (Kennedy et al., 1994). These examples are by no means an exhaustive list of candidate molecules to account for accurate nerve pathfinding, but are included to indicate the range of attractive and repulsive cues that operate during the development of the peripheral nervous system.

1.4.4 Initial Nerve-Muscle Contact

Shortly after contact between a growing motor axon and muscle fibre is established, signals are exchanged between nerve and muscle that initiate the formation and assembly of a highly differentiated presynaptic nerve terminal and a highly specialised postsynaptic apparatus (for reviews see Dennis, 1981; Hall & Sanes, 1993; Jennings & Burden, 1993). Muscle differentiation and synapse formation go on concurrently during development. In vertebrates, motor neurons do not prefer or select a predetermined site on the developing myotube; rather it appears that synapses can form on most if not all of the myotube surface (Bennett & Pettigrew, 1975). Although functional synapses form within minutes to hours after contact between developing motor nerves and myotubes (Fischbach, 1973; Anderson & Cohen, 1977), mature, fully differentiated synapses are not evident until several weeks after the first contacts are made (Nystrom, 1968; Balice-Gordon & Lichtman, 1993).

1.5 Synapse Elimination At the Neuromuscular Junction

As early as the time of myoblast fusion, outgrowing motor neurons begin to innervate muscle fibres (Bennett & Pettigrew, 1974). Initially, each motor neuron makes synaptic contacts with many muscle fibres, so that each fibre receives convergent innervation from several motor axons. In most skeletal muscles of the rat, this polyneuronal innervation begins to be lost around the time of birth (rat diaphragm: Redfern, 1970; soleus: Brown et al., 1976; lumbrical: Betz et al., 1979; EDL: Balice-Gordon & Thompson, 1988; trapezius: Colman et al., 1997). The elimination of multiple

innervation is not the consequence of axonal death or degeneration (Korneliussen & Jansen 1976; Bixby, 1981; Riley, 1981), but of the retraction of a portion of each axon's peripheral field of innervation. Evidence for this came originally from Brown et al. (1976), who showed that the neonatal loss of polyneuronal innervation was not accompanied by a reduction in the number of motor axons innervating the soleus muscle as a whole; the simplest explanation for this observation was that some terminal branches were being withdrawn from the muscle fibres.

The ultimate consequence of synapse elimination is the loss of all but one synaptic contact from each fibre. At least two factors are important in determining that the motor neurons undergo this synapse loss. First, it appears that the axonal inputs to each fibre must be competing with each other for maintenance. Direct evidence for the role of competition comes from partial denervation experiments performed using a toe muscle in the rat, the fourth deep lumbrical (4DL). The 4DL receives dual innervation (Betz et al., 1979) from the lateral plantar nerve (LPN; 6-14 motor units) and the sural nerve (SN; 0-6 motor units). Normally in adults, each muscle fibre is mononeuronally innervated, by either an LPN or an SN axon. When either the LPN or the SN is injured, the axons in the other nerve give rise to sprouts which innervate the denervated muscle fibres (Ribchester & Tuxt, 1984; Ribchester, 1988, 1993; Barry & Ribchester, 1995). In approximately 25% of cases, partially denervating the 4DL at birth by cutting the LPN results in the muscle being innervated by a single motor axon from the SN (Betz et al., 1979, 1980; Gates & Ridge, 1992). As an immediate consequence of this partial

denervation, many of the muscle fibres become singly innervated without the remaining motor axon having to retract any of its nerve terminals. It is then possible to ask whether this axon undergoes the usual developmental reduction in the number of muscle fibres it innervates. The answer from experimental studies is yes and no. No, the motor axon does not lose contact with as many muscle fibres as it would have had the partial denervation not been performed: Betz et al., (1980) showed that single remaining motor units maintained their expanded neonatal size, and did not decrease to the normal adult level. This result suggests that something about the presence of other inputs to a muscle determines the final number of fibres each motor neuron will innervate. On the other hand, there is also evidence that motor neurons remaining after partial denervation do experience a loss of innervation, supplying fewer fibres at the end of the normal period of synapse elimination than at the beginning (Thompson & Jansen, 1977; Fladby & Jansen, 1987). This reduction occurs even though some inputs are lost from fibres on which there are no other competitors. From this result, it seems that the motor neurons follow some built-in programme which causes them to reduce the number of synapses they have made in the muscle. It may be that these two mechanisms (competition and an intrinsic tendency to withdraw synaptic connections) operate together during synapse elimination.

Although the mechanisms responsible for synapse elimination are, as yet, unknown, the structural and physiological correlates of the removal of axonal inputs have been well described. The first detectable changes that appear occur at the level of the postsynaptic

membrane and take the form of a reduction in the density of AchR clustering (Balice-Gordon & Lichtman, 1993). The physiological signs of axonal removal are subtle (Rosenthal & Taraskevitch, 1977). Initially the sizes of convergent inputs, the amount of Ach released and the postsynaptic response to the transmitter are equivalent beneath convergent inputs (Balice-Gordon & Lichtman, 1993; Colman et al., 1997; Gan & Lichtman, 1998). However, the synaptic efficacies and sizes diverge shortly after the postsynaptic changes first become apparent, and continue to diverge so that the last indication that an endplate is in the process of synapse elimination is one large, strong input and one small, weak input.

1.6 The Role of Activity in Developmental Synapse Elimination

Within 5 years of Redfern's (1970) publication on synapse elimination in the rat diaphragm, studies of the role of neuromuscular activity in this process were published. In these studies, attempts were made to raise or lower the level of neuromuscular activity in neonatal animals and then to determine whether this altered the outcome of synapse elimination.

1.6.1 Treatments Reducing Activity

Tenotomy

The earliest attempts to manipulate the role of activity involved cutting muscle tendons. Electromyograms (EMG) from soleus muscles in adult rats showed that tenotomy dramatically reduced the activity of the muscle, presumably by reducing afferent

excitation to the spinal motor neurons from muscle spindles. Benoit & Changeux (1975) tenotomised soleus muscles in 4 day old rats and later examined the muscles by making intracellular recordings of the end plate potentials evoked by graded stimulation of the muscle nerve. This physiological recording method demonstrated the polyneuronal innervation of fibres by the stepwise changes in the amplitude of the end-plate potential as the stimulus intensity varied. The authors found that tenotomised muscles had higher levels of polyneuronal innervation than control muscles, at least for the two weeks subsequent to the operation. From these results, Benoit & Changeux concluded that neuromuscular activity regulated the disappearance of polyneuronal innervation. However, there are two major problems associated with these experiments. First, in the adult, tenotomy of the soleus muscle abolishes the continuous EMG activity typical of this muscle (Vrbová, 1963), but in the neonate, the tendons become reattached within a few days of their being severed (Navarrete, 1985, in Greensmith et al., 1998). Since the activity patterns of at least some neonatal muscles are different from the patterns seen in the adult (Navarrete & Vrbová, 1983), it is not at all certain that tenotomy in the neonate produces the same effects as tenotomy in the adult. Consistent with this, Riley (1978) repeated the experiments of Benoit & Changeux in rats and observed a similar delay in synapse elimination by staining end plates with silver stains and counting the number of preterminal axons entering each end plate. Riley (1976) also found that in the kitten, a delay in synapse elimination occurred following tenotomy of the soleus. However, in the adult cat, tenotomy does not appear to alter the activity of this muscle. Thus, Riley argued that it may have been wrong to

conclude that the delay in synapse elimination produced by tenotomy is necessarily due to a change in the activity of the muscle. He suggested that instead, the consequences might be due to some general effect of muscle growth.

The other problem with these early experimental methods is that the way tenotomy acts to paralyse muscles is unclear. Tenotomy may exert its effects by inhibiting electrical or mechanical activity (or some combination of the two). In a recent attempt to resolve this problem, Greensmith et al. (1998) reduced mechanical activity alone in developing rat soleus muscles, by dantrolene treatment. They found that this method of preventing excitation-contraction coupling in the muscles reduced the rate of synapse elimination, suggesting that the effects of inactivity by tenotomy may operate through mechanical mechanisms.

Spinal Transection and Deafferentation

Attempts to reduce or eliminate muscle activity have also been made by transecting dorsal roots and/or the spinal cord, thereby removing sensory input and/or descending motor input, respectively. For example, Miyata & Yoshioka (1980) transected the thoracic spinal cord in 12 day old rats and examined the effects on elimination in the soleus muscle. For the next 4 days, during which time they noted no movements of the leg, the muscles showed many more polyneuronally innervated fibres by intracellular recording than did muscles from control animals. The authors reported that movements of hindlimbs did resume, following which the muscle fibres became singly innervated.

Using the 4DL, Caldwell & Ridge (1983) examined the effects of deafferentation of motor neurons alone and in combination with spinal transection on synapse elimination. Sectioning the hindlimb dorsal roots central to the dorsal root ganglia at day 7 did not change the time course of synapse elimination, nor did it apparently alter use of the muscle. However, when the portion of the spinal cord containing the cell bodies belonging to the LPN and SN was totally isolated (by bilateral dorsal root section and transection of the cord above and below the appropriate level), polyneuronally innervated fibres were seen for a period of 10-15 days beyond the time when all of the fibres would have been expected to be singly innervated. However, these results were difficult to interpret in terms of the possibility that synapse elimination had occurred in completely paralysed muscle. Caldwell & Ridge noted that not all of their cord isolations were complete (some tail afferents remained in some animals) and that the animals were reported to display spontaneous but uncoordinated twitching movements of the leg.

Thus, while these studies demonstrate the profound effects of inactivity on synapse elimination, it is difficult to know how effective these manipulations were in completely silencing the motor axons.

Tetrodotoxin

A more potent method of preventing muscle activity is by pharmacological blockade. Tetrodotoxin (TTX) selectively blocks voltage-sensitive sodium channels and, therefore, the generation of action potentials (Miller et al., 1983). The localised delivery of TTX to a nerve will block impulse conduction at the site of application. Thus, TTX offers a method to paralyse muscle selectively. While several methods of localised TTX application have been used in the adult animal (see below, sections 1.8 & 1.9), only a few of these have been successfully applied in the neonate.

In the first study of its kind, Thompson et al. (1979) examined the effects of TTX blockade of the sciatic nerve on the elimination of polyneuronal innervation in rat soleus muscle. They applied TTX in animals aged 9-10 days, a time when every rat soleus muscle fibre is still polyneuronal innervated, but just before the time when the first singly innervated fibres begin to appear. Several observations emerged from these experiments. First, synapse elimination apparently continued for the first day or so after initiation of the block, because some singly innervated fibres appeared in the paralysed muscles. However, over the subsequent 6-7 days, high levels of polyneuronal innervation persisted in the blocked muscles while the unparalysed, contralateral control muscles underwent synapse elimination with the normal time course. These experiments argue that paralysis can prevent the appearance of singly innervated fibres in the muscle. In addition, the results show that the effects of paralysis may require a

short time to be manifest: apparently synapse elimination continues briefly after the initiation of paralysis and is halted only later.

Postsynaptic Blockers: Curare and α -Bungarotoxin

All of the above experiments employed manipulations which decreased the activity of both the motor axons and the muscle fibres. Therefore, it is difficult to conclude at which site(s) activity is important in influencing neuromuscular synapse elimination. Additional experiments employing agents which produce a postsynaptic block of transmission by binding to the AchR offer some evidence on this issue. For example, Srihari & Vrbová (1978) conducted an EM examination of the endplates in chickens during the period when muscles would normally be undergoing synapse elimination. The degree of polyneuronal innervation was estimated by counting the number of axon terminal profiles in a single section through an endplate. The administration of curare to embryos at 7 and 12 days *in ovo* blocked the decrease in the number of profiles per endplate which is normally seen at 18 days. Moreover, when the effects of the curare wore off and movements resumed, many endplates were found which had a normal number of terminal profiles. In similar physiological experiments, injection of curare into chick embryos beginning at day 6 and lasting to day 20-21 resulted in a dramatic increase in the level of polyneuronal innervation of fibres in the lateral gastrocnemius muscle (Ding et al., 1983). A morphological study on mammalian muscle similar to that of Srihari & Vrbová (1978) has also been reported by Duxson. Duxson placed strips of silicone rubber impregnated with the AchR blocker α -bungarotoxin (α -BTX;

Berg et al., 1972) alongside the soleus muscle of 10 day old rats; this treatment paralysed the limb for 2 days. The muscles were sectioned at 12 days; EM examination showed an average of 4.3 profiles per endplate in normal 12 day old soleus muscles and 6.5 profiles per endplate in animals of the same age which had been treated with α -BTX 2 days earlier. Because 5.5 profiles per endplate were seen in animals at the time of α -BTX application, Duxson concluded that the normal loss of terminals was prevented by paralysis.

Because the treatments described in all these experiments likely left motor neuron activity unaffected, these results suggest that activity in the muscle fibres themselves may be important for synapse elimination.

Presynaptic Blockade: Botulinum Toxin

The presynaptic blocking agent, botulinum toxin (BoTox) has also been employed in the study of activity and synapse elimination. Brown et al. (1982) injected BoTox into various newborn mouse hindlimb muscles. In the case of the gluteus muscle, they showed morphologically and electrophysiologically that the brief period of paralysis caused by BoTox delayed but did not ultimately prevent synapse elimination: most of the fibres became singly innervated, presumably as the muscle recovered from paralysis. However, in the tensor fasciae latae (tfl), BoTox prevented the fibres from attaining single innervation even after paralysis had worn off. The latter result was explained by an observed loss of 50-70% of tfl muscle fibres (fibre loss was much less

in the gluteus). This fibre loss produced the opposite effect on motor neuron competition as was produced by the neonatal partial denervation experiments described above. Whereas partial denervation decreased the competition for muscle fibres, the loss of muscle fibres seemed to increase this competition. At least in the case of the tfl muscle, competition apparently cannot displace motor nerve terminals if such displacement would reduce the size of the losing motor unit beyond some minimum.

Brown et al. (1981) also used BoTox to confirm the earlier observation noted by Thompson et al. (1979), that following TTX paralysis there was an initial drop in the number of polyneuronally innervated fibres, as if synapse elimination continued for a brief period after the initiation of the block. Brown et al. (1981) showed further that the amount of polyneuronal innervation subsequently reappearing in neonatal muscle was directly related to the amount of polyneuronal innervation that was present at the time of the initiation of paralysis. Following the completion of synapse elimination at about day sixteen, paralysis produced with BoTox did not result in the reappearance of multiply innervated fibres. Thus, Brown et al., defined a *critical period* occurring during the time of neuromuscular synapse elimination during which paralysis evoked the return of multiple innervated fibres and following which fibres remained singly innervated. Brown et al. suggested that the critical period might be related to the ability of the axons to respond to a muscle-secreted factor for nerve growth produced in response to paralysis. They further proposed that the ability of the motor neurons to respond to this stimulus might be related to the length of time endoneural tracks persist,

leading down to the endplates previously occupied by motor axons withdrawn in synapse elimination. For a brief period following the withdrawal of an input from a muscle fibre, these tracks persist, offering a route over which a motor axon can return to the endplate in response to a paralysis-induced release of a diffusible sprouting factor.

1.6.2 Treatments Increasing Neuromuscular Activity

The alternative approach to the induction of muscle paralysis is to increase neuromuscular activity. The first effort in this regard in the study of synapse elimination was by O'Brien et al. (1978). These authors placed stimulating electrodes around the sciatic nerves of rat pups 6-7 days old and administered stimulation at 8Hz to the nerves for 4-6 hours per day for 2-4 days. At the end of stimulation, levels of polyneuronal innervation in the stimulated and in contralateral control muscle were established using intracellular recording and endplate morphology. The level of polyneuronal innervation in the stimulated muscles decreased from the control levels of 84-87% of the fibres to 45-60% following 2-3 days of stimulation. From these results, it was concluded that increased activity accelerated the elimination of synapses. One difficulty in these experiments is controlling for nonspecific effects of the stimulation. O'Brien et al. also observed that their stimulation accelerated the elimination of synapses from the contralateral muscle evoked by a crossed extension reflex, so the possibility that stimulation might be affecting elimination via a systemic effect (for example increased levels of stress hormones) could not be excluded. Thompson (1983) also showed that imposing a stimulation regime on nerves accelerated the time course

of synapse elimination. However, the effectiveness of externally applied stimulation in altering synapse elimination was dependent on the pattern of the stimuli delivered to the nerves: brief stimulus trains of 100Hz were effective in producing the acceleration of input loss, but the same number of stimuli presented continuously at 1Hz were not.

1.7 Muscle Reinnervation in Adults

When peripheral nerves are transected, the isolated distal portions of the axons degenerate (Waller, 1852). The first part of the motor axon to degenerate is the presynaptic terminal, which in rodents, breaks down completely within less than 24 hours (Miledi & Slater, 1970). If the nerve is damaged by crushing, so that the basal lamina around each axon remains intact, the axons regenerate and rapidly grow back to the muscle. In most cases, the regenerating axons reinnervate the muscle precisely at the sites of the original neuromuscular junction (Letinsky et al., 1976; Rich & Lichtman, 1989). These regenerated neuromuscular junctions look and perform much like those seen during normal development, so reinnervation of adult muscle provides an accessible setting in which to analyse synaptogenesis.

Studies of partial denervation (whereby a portion of the nerve supply to a muscle is injured) show that axotomised nerve terminals degenerate and withdraw from muscle fibres and intact, uninjured axons sprout to occupy the territory that has been left vacant (Brown et al., 1981; Barry & Ribchester, 1995). Unlike developmental synaptogenesis, this compensatory innervation seems to be directed by Schwann cells, which sprout

dramatically following denervation (Reynolds & Woolf, 1992). Both regenerating and intact axons grow along the sprouted Schwann cell tracks in order to reach denervated synaptic sites (Son & Thompson, 1995a, 1995b). Often, when regenerating axons return to the muscles they once innervated, synaptic contacts are made with fibres that may already be occupied by sprouts, and so some fibres become polyneuronally innervated (Hoffman, 1953; Guth, 1962; McArdle, 1975; Brown & Ironton, 1976, 1978; Ribchester & Tuxt, 1984; Ribchester, 1988; Barry & Ribchester, 1995). The subsequent synapse elimination that occurs very closely resembles that seen during neonatal development, described above. Briefly, AchRs (Rich & Lichtman, 1989) and other anchoring and clustering molecules (rapsyn, utrophin, phosphotyrosine, dystrophin and syntrophin; Culican et al., 1998) disappear from the postsynaptic membrane. Presynaptic changes and the removal of Schwann cell processes from the endplate follow very closely behind these postsynaptic changes. Schwann cell processes appear to lag slightly behind the time that AchRs and associated molecules disappear, but are removed shortly before the nerve terminal boutons are lost from the endplate (Trachtenburg & Thompson, 1996,1997; Culican et al., 1998).

1.8 Effects of Paralysis on Synapse Elimination in Reinnervated Muscle

TTX Treatment

An early investigation into the effect of activity on synapse elimination following reinnervation (Tuxt, 1983) confirmed developmental observations. That is, paralysis was found to enhance the proportion of polyneuronally innervated muscle fibres. In this

study, Taxt (1983) performed a full denervation of the 4DL by crushing both the LPN and SN and then blocked nerve conduction in both nerves by chronically superfusing TTX to the sciatic nerve (both the LPN and SN arise from separate branches of the sciatic). He found that synapse elimination was slowed, or possibly abolished using this paradigm. Taxt was not able to distinguish between these two alternatives, because it was possible that synapse elimination was entirely halted, or merely exceeded by synapse formation in the muscles.

More recently, Barry & Ribchester, (1995) examined the effects of a period of paralysis on subsequent synapse elimination after the resumption of activity. The authors performed a partial denervation of the 4DL by crushing the LPN and blocked activity in the sciatic nerve with TTX (following recovery from LPN crush). Immediately following the resumption of activity in the muscles, approximately 60% of fibres were polyneuronally innervated. During the next two weeks, levels of polyneuronally innervation fell to 30%, but remained stable thereafter (the authors examined muscles up to 8 weeks following the resumption of activity). Like Brown et al. (1981), these results suggest that activity may act during a critical period to influence the retention of persistent polyneuronal innervation.

However, another study suggests that activity may not be necessary at all for synapse elimination during reinnervation of the 4DL muscle. In these experiments, Ribchester (1993) performed a minor partial denervation by crushing the SN. The LPN, supplying

the majority of muscle fibres in a given 4DL (see above), is usually capable of sprouting to compensate for SN denervation (Ribchester, 1988). Thus, in this paradigm, regenerating SN axons returned to endplates that were already innervated by the LPN. Just before SN axons returned to the muscles, Ribchester blocked activity in both nerves with TTX. Using electrophysiological measurements, he found that at some endplates, the SN displaced LPN inputs, suggesting that activity is not required for synapse elimination in all cases.

1.9 Differential Activity During Synapse Elimination

The discussion of activity thus far has implied that muscle fibre activation determines synapse elimination by influencing the level of competition among the nerve terminals on each muscle fibre. Although no implication has been made that activity might somehow be able to determine which particular axonal input is retained at the end of elimination, it has been suggested that activity does fulfil this role. This question has attracted considerable interest, largely owing to the work by Hubel & Wiesel (1965) showing that the input neurons in the visual cortex are influenced during an early critical time period by visual experience. It seems that dissimilar visual experience through the two eyes leads to an increase in the number of cells which can be driven through the more experienced eye, at the expense of cells driven by the deprived eye. It was therefore of interest to know whether motor axons which are more active have a competitive advantage in synapse elimination over motor axons which are less active.

1.9.1 Treatments For Differentially Reducing Activity

TTX

Because of the difficulty in manipulating the activity of a portion of the innervation to a muscle in a small, neonatal animal, the earliest exploration of this question made use of the synapse elimination occurring following reinnervation of the 4DL muscle (Ribchester & Tact, 1983). Using TTX to block activity in the LPN following recovery from SN and LPN crush, it was found that the active SN axons eliminated twice as many fibres as in control muscles (which had not been subjected to activity block). These results suggested that inactive axons were preferentially displaced from fibres by active motor terminals.

In a related set of experiments, Ribchester & Tact (1984) performed a minor partial denervation of the 4DL (SN crush) and blocked activity in the LPN with TTX during reinnervation. This paradigm allowed the authors to ask whether intact, but inactive inputs were still disadvantaged when pitted against regenerating axons. Consistent with the results described above, the active inputs appeared to have a competitive advantage during reinnervation: the SN motor units in the crush/block muscles were enlarged compared with control 4DLs, which were subjected to SN crush only.

Finally, Ribchester (1998) performed a major partial denervation of the 4DL (LPN crush) and blocked activity with TTX in the LPN during reinnervation. The sizes of LPN motor units in these muscles were compared with LPN motor units in 4DLs which

had been subjected to LPN crush only. Ribchester found that the regenerating axons innervated fewer fibres when they were blocked with TTX, supporting the idea that activity confers a competitive advantage on inputs during reinnervation.

All of these results argue that there is a mechanism at the neuromuscular junction which favours the retention of the more active nerve terminal during synapse elimination. However, there is also evidence for the opposite conclusion: that less active inputs have a competitive advantage. Callaway et al. (1987, 1989) blocked activity with TTX in one of three ventral roots innervating rabbit soleus muscle. These authors found that axons in which electrical activity was blocked displayed larger motor units than axons from nerve roots in which activity was not blocked.

α -BTX

The selective loss of AchRs and nerve terminal boutons during synapse elimination (described above, section 1.5) has shown to be activity dependent in a competitive manner. This was demonstrated by Balice-Gordon & Lichtman (1994), who focally applied α -BTX to portions of AchR plaques in normal, adult sternomastoid muscles. The desynchronised activity that resulted led to the selective loss of AchRs from the blocked portions of the receptor plaque, and withdrawal of the overlying nerve branches in that area. In contrast, blockade of the whole junction had no effect on selective elimination, suggesting that inactive regions are at risk for elimination only when some threshold level of activation remains in the rest of the junction. This paradigm differs

from polyneuronally innervated endplates during development and after reinnervation in the respect that Balice-Gordon & Lichtman's endplates comprised one active region and one inactive region, rather than two non-synchronously active regions. Nonetheless, the authors argue that the results indicate a competitive advantage for more active inputs at endplates.

1.9.2 Treatments For Differentially Elevating Activity

Nerve Stimulation

Ridge & Betz (1984) also used the 4DL to examine the effects of differential activity on neonatal synapse elimination. They exposed and stimulated either the LPN or the SN in week old rats every day for 4 hours per day. After 5-6 days of stimulation, they estimated the sizes of individual motor units by recording the tensions evoked by stimulation of single motor axons. The differences observed between stimulated and non-stimulated motor units were small and the samples were found to be statistically significant only when comparisons were made for motor units within each animal. Nonetheless, the authors found that stimulated motor units tended to increase in size at the expense of those units which were not stimulated.

Activity has also been shown to confer an advantage on synapse preservation in cell culture. Stimulating one of two explanted chick skeletal muscle cells resulted in the elimination of nearly all functional contacts from the unstimulated ganglion by 1-7

days, and the polyneuronal inputs from the stimulated ganglion became reduced to mononeuronal inputs (Magchielse & Meeter, 1986).

However, contradictory results have also been described using a cell-culture assay. Nelson et al. (1993) made a three-compartment chamber with mouse muscle in the centre and either spinal motor neurons or superior cervical ganglion neurons in the side chambers. Axons grew from either side into the central compartment and, in many cases, coinnervated muscle fibres. By monitoring muscle contraction in response to stimulation of axons from either side and by repeated electrophysiological recording, synapse elimination was established. Consistent with the results of Callaway et al (1987, 1989), described above, Nelson et al. found that synapse elimination was accelerated by repeated tetanic stimulation of one or both sets of axons, but stimulated inputs to fibres were just as likely to be eliminated as unstimulated ones. In fact, bilateral stimulation caused the most synapse elimination. Because identified junctions were not followed over time, and subthreshold junctions would have been missed in the functional assay, it is not possible to be as specific as one might like about the relative efficacy of different inputs to a cell, the time course of activation, or the completeness of elimination. Nonetheless, these results show that additional mechanisms are required to understand activity-dependent synaptic modulation in the developing nervous system.

1.10 Summary

It is clear that activity has profound influences on the time course and outcome of neuromuscular synapse elimination. However, despite a great deal of work, there is still little understanding of the ways activity exerts its influences. In particular, the above review highlights two contradictions with respect to the centrality of activity in synapse elimination. First, the majority of evidence suggests that differences in amounts and patterns of activity lead to synapse elimination, so that wherever it occurs, polyneuronal innervation will be transformed to mononeuronal innervation. However, other studies show that polyneuronal innervation persists in skeletal muscle. Second, it is widely accepted that paralysis creates an environment in which synapse elimination does not occur. This idea is a corollary of the first: if the muscle fibre cannot sense differences in the timing of activation, synapse elimination will not be triggered. However, there are studies which show that inactive inputs do engage in synapse elimination. Thus, the necessity and sufficiency of activity remain to be established. The experiments presented here were designed, in part, to test these two conditions.

Chapter 2: Materials & Methods

2.1 General

The protocols discussed in this section are common to all chapters. Procedures specific to separate chapters are detailed in subsequent sections.

2.1.1 Animals and Animal Care

Female Sprague Dawley (SD) rats were used for all experiments. Animals were housed in 250cm² cages with an internal height of 20cm, under standard animal house conditions of light and temperature. Rats were caged in groups, at a maximum of six animals per cage, with free access to food and water at all times.

2.1.2 Aseptic Procedures

All surgical procedures were licensed by the UK Home Office and carried out under aseptic conditions. All gowns, tissues, cloths, and instruments used in operations were sterilised. Surgical caps and masks were worn throughout all procedures. All gloves, sutures and chronically implanted materials were sterilised by the manufacturer. The appropriate areas of the animal were shaved and swabbed with antiseptic (Betadine; Seton Healthcare Group, Plc.).

2.1.3 Operative Procedures

Anaesthetic

Animals were placed in a lidded Perspex chamber with a perforated floor. To induce anaesthesia, Halothane (Rhône-Poulenc Rorer, Ltd.) at 5% in N₂O/O₂ (1:1) was delivered by inhalation, using a Fluovac System (International Market Supply). Animals were then transferred to the lab bench and anaesthetic was delivered via a

face mask (Fig 2.1). Halothane levels were reduced to 2.5% in N₂O/O₂ (1:1) to maintain deep anaesthesia. Throughout the operation, the level of anaesthetisation was monitored by ensuring the absence of (1) blinking in response to tickling the eyelashes and (2) withdrawal reflexes to pinches of the toes and tail with blunt forceps.

Nerve Crush

Under halothane anaesthesia, the skin and fascia overlying the appropriate nerve were opened. Using sharp dissection scissors the nerve was exposed and crushed for 30 seconds using fine forceps. The wounds were closed and sutured using 6/0 silk suture (Ethicon).

Chronic Implants

Under halothane anaesthesia, the right sciatic nerve was exposed in the thigh and a laparotomy was performed (Fig 2.1). An osmotic minipump (Alzet 2002; Charles River, UK Ltd.) containing TTX in citrate buffer (Alomone Labs, 500 µg/ml in sterile saline plus 200 µg/ml ampicillin) was implanted intraperitoneally. The outlet of the minipump was connected to narrow bore platinum cured silicone rubber tubing (i.d. 0.5mm, o.d. 0.93mm; British Drug Houses, [BDH]) which terminated in a 1cm long cuff constructed of wider silicone tubing (id.2mm; BDH). The narrow tubing was threaded subcutaneously between the sites of the laparotomy and sciatic nerve exposure. The cuff was placed around the sciatic nerve. The tubing, but not the cuff, was also filled with TTX. Internal and external wounds were sutured with

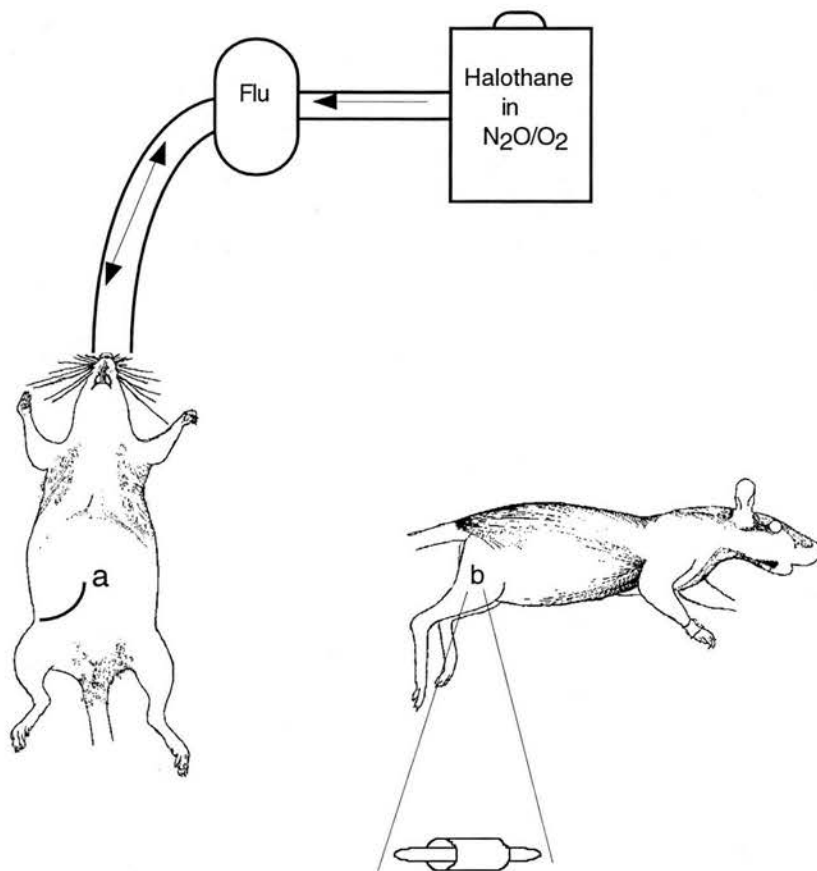


Fig 2.1 Surgical and anaesthetic procedures. Following deep anaesthetisation, the animal was placed on its back, and Halothane/gasses were delivered through tubing, which terminated in a mask. The mask was placed loosely around the nose and mouth with a continuous flow of gasses escaping past the animal's nose. Recycled gasses were captured and filtered with a Fluosorber (Flu). Osmotic minipumps were implanted in the peritoneum (marked a, in the diagram) connected subcutaneously via silastic tubing (indicated by the curved line) to the cuff implanted around the sciatic nerve. The letter b designates the location of the incision made to expose the sciatic nerve. The enlarged window depicts a length of sciatic nerve ensheathed, but not compressed by the cuff.

7/0 and 6/0 silk suture, respectively. This operation is referred to as a “TTX implant” or “TTX activity block”.

Behavioural Indicators of Limb Paralysis

Paralysis of the right hind limb was monitored daily by observing (1) the complete absence of toe-spreading reflexes when animals were gently lifted by the tail, and (2) the absence of withdrawal reflexes in response to firm pinches applied to the toe pads with blunt forceps (Fig 2.2).

2.1.4 Animal Sacrifice

All animals were killed by cervical dislocation, in accordance with UK Home Office licensing regulations.

2.1.5 4DL Nerve-Muscle Preparations

Hind limbs were removed and 4DL muscles with their intact dual nerve supplies were dissected in mammalian physiological saline (concentrations in mM: NaCl 120; KCl 5; CaCl₂ 2; MgCl₂ 1; NaH₂PO₄ 0.4; NaHCO₃ 23.8; D-glucose 5.6), bubbled with O₂/CO₂ (95%/5%). Nerve-muscle preparations were pinned with fine minuten pins (0.2mm diameter; Interfocus Ltd) in a dish lined with Sylgard (Dow Corning) and perfused with the oxygenated physiological saline.

2.1.6 Visualisation of the Presynaptic Specialisation with Styryl Dyes

Nerve terminal boutons were stained with the vital aminostyryl dyes FM1-43 and RH414 (Molecular Probes). FM1-43 (10mM in distilled H₂O) was made up to a

final concentration 2 μ M in oxygenated Ringers. Preparations were bathed in the Ringers/FM1-43 solution and the SN was attached via a suction electrode to a pulse stimulator (Harvard). Pulses were delivered to the SN at 20mV/15Hz for 10 minutes. The preparation was washed in the FM1-43 on a platform shaker (BDH) for 10 minutes and bathed in oxygenated physiological saline for 30 minutes. The preparations were then bathed in a 20 μ M solution of RH414 (in oxygenated Ringers; from an initial concentration of 10mM made up in distilled H₂O). The LPN was stimulated and washed using the same paradigm described above, for the SN.

Nerve terminals were viewed in a fluorescent microscope (CF Fluorescent, Nikon) using a Zeiss 40x water immersion objective, a mercury lamp (Nikon HBO-100W/2) and a fluorescein excitation/emission filter block (400-440nm excitation and 510-520 emission filters; Nikon). Images were captured with a colour chilled 3CCD camera (Hamamatsu) to an Apple Macintosh computer (Quadra) using OpenLab (Improvision) and Adobe Photoshop software. Areas occupied by SN and LPN terminal boutons were measured from binary masks judged to exactly overlap the respective areas of FM1-43 and RH414 staining. The microscope and camera were mounted on a stage support (Microinstruments M2B) attached to an anti-vibration table (Newport). Images were analysed and enhanced using OpenLab, Adobe Photoshop, Microsoft Excel, and ClarisWorks software and printed on a Kodak ColorEase printer.

2.2 Visualisation of the Presynaptic Specialisation Using Immunocytochemistry (Chapters 3 and 4)

Muscles were fixed in 4% paraformaldehyde (Sigma Chemical) for 15mins and AchRs were labelled with rhodamine-conjugated α -BTX (TRITC- α -BTX; Molecular Probes; see below, section 2.3). After washing and permeabilising, the preparations were tagged with primary monoclonal antibodies directed against medium weight neurofilaments (nf; 165kDa) and synaptic vesicle proteins (SV2). All primary antibodies were received from The Developmental Studies Hybridoma Bank. Visualisation of axons and nerve terminals was achieved by the addition of a fluorescein-conjugated sheep anti-mouse secondary antibody (SAPU, Law hospital, Carlisle, Scotland, UK). See page 41 for detailed protocol.

2.3 Visualisation of the Postsynaptic Specialisation (For Vital and Immunocytochemical Staining; Chapters 3 and 4)

Muscles were pinned in a Sylgard-lined dish and placed in a 5 μ g/ml solution of TRITC- α -BTX (in oxygenated Ringers; stocks were dissolved in distilled H₂O). The dish was placed on a platform shaker for 20 minutes; the α -BTX was discarded and muscles were washed in oxygenated physiological saline for 20 minutes and viewed under the fluorescent microscope using a rhodamine excitation/emission filter block (Nikon).

2.4 Intracellular Electrophysiological Measurements (Chapters 4 and 5)

Nerve-muscle preparations were placed in a Sylgard-lined dish and perfused continuously throughout recording with oxygenated physiological saline. Muscle action potentials were blocked with μ -conotoxin (2 μ M for 20 mins; Scientific

Marketing Associates). The SN and LPN were drawn up into separate suction electrodes. Supramaximal square wave pulses of 0.1ms were delivered from battery powered isolation units (Digitimer DS2A). The timing of pulse delivery was regulated using a Neurolog system (Digitimer, model D4030).

Intracellular recordings were made with glass microelectrodes (1.5mm o.d. x 0.86mm i.d.; Clark Electromedical Instruments) pulled on a Flaming/Brown Micropipette Puller (Model P.87/PC, Sutter Instrument Co.) and back-filled with 3M KCl. The resistance of the electrodes was between 20-40 M Ω . Electrodes were attached to the headstage preamplifier (Duo 773 Electrometer) by an electrode holder (World Precision Instruments Ltd.), containing a piece of chlorided silver wire. The reference electrode was a chlorided silver wire in contact with the physiological saline inside the recording dish. Muscle fibres were impaled by advancing the microelectrode with a micromanipulator (Leitz).

The command pulse and recorded response were amplified and displayed simultaneously on a storage oscilloscope (Tektronix) and the membrane potential was monitored on a digital display (Duo 773 Electrometer).

Intracellular signals were digitised using a CED 1401+ (Cambridge Electronic Design) interface and a personal computer (Dell). Quantal contents were measured using the variance and (where appropriate) failures methods. The EPP amplitudes and quantal contents were calculated after normalising the resting membrane potential to -80mV, assuming 0mV as the reversal potential for the Ach-induced ion

current (Magleby & Stevens, 1972). The normalised values were corrected for non-linear summation using the correction formula derived by McLachlan & Martin (1981): $EPP' = EPP/[1-f(EPP/E)]$, where EPP' is the corrected EPP amplitude and E is the difference between resting membrane potential and the reversal potential for Ach. The value of f (a constant dependent on the duration of transmitter action relative to the membrane time constant) was set to 0.8 (McLachlan & Martin 1981). All calculations were made using WCP software, supplied by Dr. J. Dempster (University of Strathclyde, Glasgow).

2.5 Intracellular Recordings From Identified Neuromuscular Junctions (Chapters 4 and 5)

Images of the endplate were captured with fluorescent light. A microelectrode was positioned near an endplate under visual control, using a combination of low and fluorescent light. In some cases, electrodes were filled with a mixture of 3% Lucifer Yellow (made up in 0.1M LiCl; Molecular Probes), in order to facilitate the location of the tip of the microelectrode (Fig 2.3). Images of the endplate were captured again with fluorescent light after impaling the muscle fibre and recording synaptic responses.

2.6 α -BTX Injections (Chapter 3)

Unlabelled α -BTX (Molecular Probes) was made up in sterile “non-sprouting buffer” (recipe provided W.J. Thompson, personal communication):

20mM Sodium Acetate
100mM Arginine
1% Mannitol
100mM Sodium Sulphate
0.1% BSA

pH, 6.9

to a final concentration of 500µg/ml. Under halothane anaesthesia, 5µl was injected into the toe pad overlying the 4DL muscle of the right hind limb using a 25µl syringe (Hamilton).

2.7 Statistics

Unless stated otherwise in the text, the following statistical tests were performed, using GraphPad (Instat):

- i) Unpaired, nonparametric t tests (Mann Whitney).
- ii) Nonparametric one way analysis of variance (ANOVA), with Dunn's *post hoc* tests.
- iii) Correlation and regression analysis for linear relationships (Pearson's r).

Throughout the results section, all parameters are expressed as mean \pm standard error of the mean (SEM).

Immunocytochemistry for Neurofilament, Synaptic Vesicles and Ach Receptors

All steps done on a platform rotator.

Day 1:

1. Fix 4DL preparation for 15 minutes in 4% Paraformaldehyde:
4g paraformaldehyde (Sigma)
100 μ l 10M NaOH
100ml 1x Phosphate Buffered Saline (PBS; Sigma)
heat to 60°C shaking/stirring continuously
cool to room temperature
2. Wash 3 times for 10 minutes in 1xPBS, discarding 1xPBS between washes.
3. Incubate in 5 μ g/ml TRITC- α -BTX for 20 minutes.
4. Wash 3 times for 10 minutes in 1xPBS, discarding 1xPBS between washes.
5. Plunge preparation into -20°C methanol for 5 minutes.
6. Wash 3 times for 10 minutes in 1xPBS, discarding 1xPBS between washes.
7. Incubate preparation in the following “preblock” solution for 30 minutes:
10ml 1x PBS
30 μ l Triton X (Sigma)
.02g BSA (Sigma)
.01g Na Azide (Sigma)
8. Incubate in the following “primary” solution overnight:
4ml preblock solution
12 μ l nf antibody (diluted 1:250)
8 μ l SV2 antibody (diluted 1:500)

Day 2:

9. Wash 3 times for 10 minutes in preblock solution, discarding the solution between washes.
10. Incubate preparation in the following “secondary” solution for 1 hour:
4ml preblock solution
30 μ l FITC sheep-anti-mouse antibody (diluted 1:100)
11. Wash 3 times for 10 minutes in 1xPBS, discarding 1xPBS between washes.
12. Mount the preparation on a slide, cover with Vectashield (Vector Laboratories Inc.) and coverslip.

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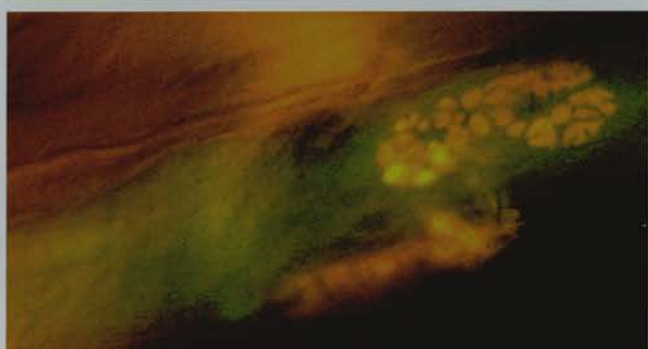
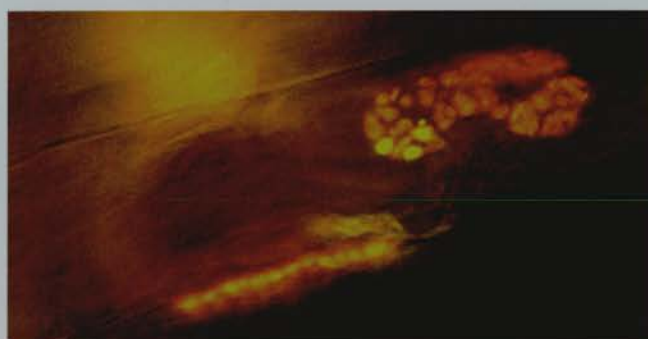
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Fig 2.2 TTX superfusion to the right sciatic nerve paralyses the lower limb, indicated by the lack of toe spreading reflexes. (a): Upon recovery from anaesthesia immediately following TTX implant, rats failed to spread the toes of the right hind foot; spreading reflexes in the left foot were unimpaired. Scale bar is 1cm. (b): In an unoperated control rat, the spreading reflexes of both hind feet were equivalent, and unimpaired. Scale bar is 1.75cm.



Fig 2.3 Intracellular recordings from identified neuromuscular junctions in the 4DL were made under visual control. An endplate of interest was positioned in the centre of the field of view (top panel). A microelectrode was positioned within 100 μ m of the endplate and the muscle fibre was penetrated (middle panel). Following recording, the electrode was removed and the fibre was imaged again. In this example, the presence of LY staining in the muscle fibre helped to confirm the accuracy of the impalement; note the diffusion of the dye in the middle panel, and that the presence of LY is exclusively within the muscle fibre of interest. Scale bar is 30 μ m.



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Chapter 3:
Activity-Independent Synapse Elimination

3.1 Introduction

Studies of neuromuscular development (Thompson et al., 1979; Brown et al., 1982; Callaway et al., 1987, 1989) and reinnervation (Ribchester, 1993) have suggested that inactive axons are capable of displacing other inactive and/or active axons from polyneuronally innervated muscle fibres. These studies contrast sharply with a wealth of experimental evidence suggesting that synapse elimination does not occur unless neuromuscular junctions are used (Benoit & Changeux, 1975; Riley, 1976, 1978; Brown et al., 1981, 1982; Duxson, 1982; Tøxt, 1983; Balice-Gordon & Lichtman, 1994). The reason for this contradiction is unclear, but may rest with the experimental designs used in studies of activity-independent synapse elimination. These studies have primarily employed electrophysiological techniques, without parallel anatomical/synaptic imaging. Physiological measurements alone do have some disadvantages for the analysis and interpretation of data. For example, using twitch tension measurements, the size of motor units can be only crudely determined. In addition, it is not possible to obtain information on inputs which are subthreshold for activation of the muscle fibre. On the other hand, intracellular recording can reveal the presence of multiple axonal inputs to a muscle fibre, but an accurate determination can be difficult if the number of inputs is substantial, or their resistance properties are similar. Finally, the intracellular method involves recording from a sample of fibres in a muscle, and there is no way to determine whether the sample is representative of the muscle as a whole. Likewise, relying only on anatomical methods to determine the innervation patterns of endplates can be misleading. Although histology may reveal the number of axons supplying an

endplate, an indication of the dynamic nature of synaptic rearrangement over time can be difficult to establish.

These methodological criticisms are in no way intended to negate the results of earlier studies, but are noted in order to demonstrate that previous findings could be complemented and extended with imaging techniques that are currently available. For example, styryl dyes which fluoresce at different wavelengths can be used to selectively label nerve terminal boutons, revealing the presence of mono- and polyneuronally innervated endplates throughout the whole muscle (Lichtman et al., 1985; Betz et al., 1992; Barry & Ribchester, 1995). The experiments described in this chapter were conducted, in part, to determine if vital synaptic imaging could compensate for some of the weaknesses of purely physiological or anatomical approaches to the question “is activity necessary for synapse elimination?”.

A second, and perhaps more important criticism of earlier studies is that the methods used may have been insufficient to abolish neuromuscular activity entirely. In all cases, TTX was used to prevent evoked potentials. In skeletal muscle, TTX interrupts the generation of action potentials by blocking voltage activated sodium channels (Miller et al., 1983). However, TTX does not block calcium channels (Llinàs et al., 1981), and there is evidence that spontaneous neurotransmitter release is mediated by calcium entry to the nerve terminal via calcium channels. For example, Grinnell & Pawson (1989) showed that TTX had no effect on MEPP frequency at the frog neuromuscular junction, suggesting spontaneous release is not dependent on calcium entering the nerve terminal through TTX-sensitive sodium

channels. Regardless of the mechanism of spontaneous release, TTX block does not prevent MEPPs. Thus, the possibility remains that there is a role for spontaneous activity in the initiation and action of synapse elimination, at least under conditions of evoked activity blockade.

In order to address this possibility, evoked and spontaneous activity were simultaneously abolished in newly reinnervated 4DL muscles. The hypothesis tested was that axons would not be able to engage in synapse elimination in the absence of all activity. A combination of vital and immunocytochemical staining was chosen as the method of investigation so that patterns of innervation could be directly observed throughout the entire muscle. In addition, the styryl dyes would reveal the presence of active nerve terminal boutons, and immunocytochemical staining could be used to confirm the distribution of synaptic inputs, regardless of their being active or inactive.

3.2 Experimental Design and Methods

The paradigm used here was based on a design by Ribchester (1993). Fourth deep lumbrical muscles were subjected to bilateral minor partial denervations by crushing all SN axons (Fig 3.1). Previous studies show that 1-2 weeks after partial denervation of the 4DL, injured axons have fully degenerated and intact axons have sprouted maximally (Ribchester & Tact, 1984; Ribchester, 1988). Fourteen days after SN crush, activity was blocked in the right hind limb for a further 14 days with a TTX implant. Animals were sacrificed on day 14 of TTX treatment, so that activity did not resume in the muscles. Thus, 4DL muscles that had been fully

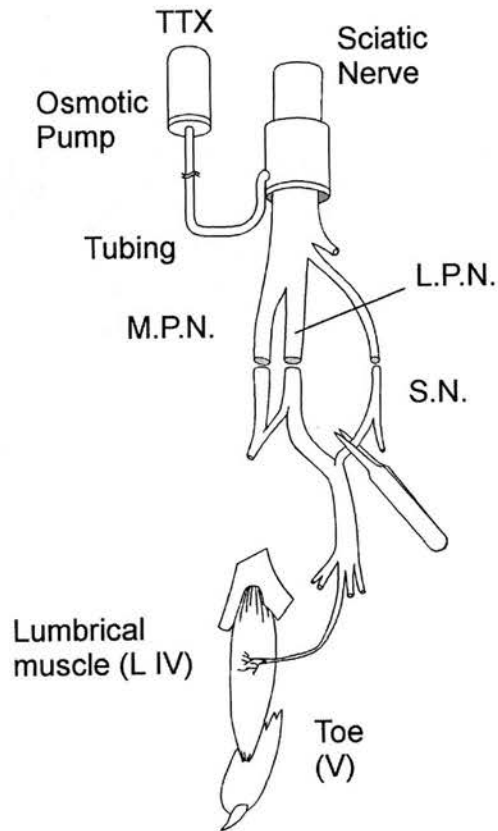


Fig 3.1 The LPN and SN each arise from separate branches of the sciatic nerve and form an anastomosis at the ankle. From here, the two nerves travel together, in a single muscle-nerve, to provide dual innervation to the 4DL. The medial plantar nerve (MPN) innervates other muscles in the foot. *Experimental design:* the forceps indicate the location of the SN crush: as close as possible, but avoiding damage to, the anastomosis. Fourteen days after the nerve injury, an osmotic minipump was implanted in the peritoneum, and TTX was continuously superfused to the sciatic nerve via silastic tubing and a cuff. *Image reproduced, with permission, from Ribchester (1993).*

innervated by the LPN were presented with regenerating SN axons in the absence of activity. Any subsequent exclusive reinnervation of fibres by SN axons would indicate activity-independent synapse elimination had taken place.

In the first series of experiments, animals were sacrificed 3 days after bilateral SN crush so that the early time course of SN degeneration and LPN sprouting could be established. Immunocytochemistry was used to determine the extent of innervation.

In the second series of experiments, animals were sacrificed 14 days after bilateral SN crush. Immunocytochemical staining was used to establish (1) whether regenerating SN axons had returned to 4DL muscles and successfully competed with LPN axons for innervation of the fibres *before* the imposition of the activity block, and (2) whether there were any endplates that failed to attract innervation from the LPN after SN crush, and were vacant when regenerating SN axons returned.

In the third set of experiments, FM1-43, RH414 and immunocytochemical staining were used to determine the presence of any endplates exclusively occupied by regenerating SN axons after SN crush and TTX activity block.

In the final experiments, activity was blocked pre- and postsynaptically for 14 days by TTX treatment in conjunction with daily injections of unlabelled α -BTX (here called "TTX/ α -BTX block"), which binds irreversibly to the AchR (Berg et al., 1972). The repeated injections were necessary to compensate for the increased synthesis and turnover of junctional AchRs which accompanies muscle paralysis (Salpeter & Marchaterre, 1992; Shyng & Salpeter, 1990). Injections were made into

the right foot only, at the same time every 24 hours, and animals received an injection on the day of the acute experiment (day 14), before sacrifice. Styryl dyes were used to determine the innervation patterns of 4DL endplates.

3.3 Controls

3.3.1 Contralateral Controls

As stated above, 4DL muscles were partially denervated bilaterally 14 days before TTX implants, in the TTX and TTX/ α -BTX experiments. The 4DL muscles from the left feet of rats in the two experimental conditions were grouped together to provide contralateral control data (28 days recovery from SN crush). Three control muscles were from animals in the TTX/ α -BTX experimental group, and one control muscle was from an animal in the TTX block group.

3.3.2 Osmotic Minipump/Sciatic Cuff Implants

Saline-containing osmotic minipumps/cuffs were implanted in two rats, matched for age and weight to animals which received TTX treatment. These rats, called “sham implanted controls”, were sacrificed 3 days after the saline implant. LPN and SN terminal boutons were both stained with FM1-43 and receptors were stained with TRITC- α -BTX. The purpose of these experiments was to determine whether the cuff routinely damaged axons in the sciatic nerve, resulting in denervated muscle fibres and/or an impaired regenerative capacity by some axons. Nerve terminal withdrawal normally occurs within 12-24 hours of axonal injury (Waller, 1852), so any vacant or partially occupied endplates should have been apparent 3 days after the sham implant.

3.3.3 α -BTX Injections

Precision and Accuracy of a Single α -BTX Injection

Control experiments were conducted to verify that the injected α -BTX reached the receptors of the 4DL. Rhodamine-conjugated α -BTX was injected bilaterally (1 μ g/ μ l made up in distilled H₂O) in 1 control, unoperated rat using the same technique used for administering unlabelled α -BTX to the experimental animals. Twenty-four hours later, the animal was sacrificed and 4DL muscles were dissected and placed under the fluorescent microscope to see if any fluorescently labelled α -BTX was visible at the endplates.

Consistency of Unlabelled α -BTX Injections

To assess the degree of AchR block on the day of the acute experiments, *in vitro* intracellular recording was performed in one TTX/ α -BTX blocked muscle. The goal was to detect MEPPs and evoked responses to nerve stimulation from individual fibres. In two other TTX/ α -BTX blocked muscles, an attempt was made to stain receptors with TRITC- α -BTX; any rhodamine positive staining would indicate the location of receptors that had failed to be blocked by injected α -BTX.

Patency of the AchR Block Between Injections

Following denervation or paralysis, the half-life of receptors at the endplate decreases from 8 days (Salpeter & Marchaterre, 1992) to 3 days (Shyng & Salpeter, 1990). New receptors synthesised while the muscle is paralysed turn over with a half life of 24 hours (Shyng & Salpeter, 1990). Control experiments were performed to

ensure that newly synthesised AchRs inserted into the muscle fibres between injections did not compromise the integrity of the activity block.

Activity-blocked and contralateral control muscles were prepared in one animal as described above, with a modification in the injection regime: unlabelled α -BTX was injected into the right foot every day for 12 days. On day 13, the animal received bilateral injections of TRITC- α -BTX. The rat was sacrificed on day 14 and both 4DL muscles were examined under the fluorescent microscope for any evidence of AchR staining.

Finally, activity-blocked and contralateral control muscles were prepared in 2 animals, but unlabelled α -BTX injections were given to the right feet every day for 13 days. On day 14, EMG recordings were made from the blocked and contralateral control muscles of one rat by taking extracellular recordings with a hook electrode from SN and LPN motor units. The 4DL muscles from the other rat were dissected, stained with TRITC- α -BTX and examined under the fluorescent microscope.

Results

3.4 Efficacy of the Activity-Blocking Paradigms

3.4.1 Patency of the TTX Block

TTX administration to the right sciatic nerve produced a paralysis of the right hind limb that was noticeable as soon as the rats recovered from anaesthesia. Rats (1) failed to use the right hind limb when walking, (2) showed no withdraw reflex of the right leg when the toe pads were pinched with blunt forceps and (3) did not spread the toes when lifted by the tail. All of these behavioural indicators were present in the contralateral hind limb. Every rat which showed marked leg paralysis on the TTX side also showed sciatic nerve block, upon sacrifice and dissection. In all animals included in the study, there was no detectable muscle response to cutting the sciatic nerve *in situ* above the cuff. Cutting the sciatic nerve below the cuff elicited vigorous muscle contractions.

3.4.2 Mechanical Effects of Implanted Cuffs

Sham-implanted controls did not exhibit any clinical signs of paralysis in either hind limb over the three day period of observation (N=2 animals; n=2 muscles). In addition, neither showed evidence of vacant or partially occupied endplates (n=286 fibres assessed in total), when 4DL muscles were examined three days after the sham implant (Fig 3.2).

3.4.3 α -BTX Injections

Precision and Accuracy of a Single α -BTX Injection

Twenty-four hours after TRITC- α -BTX was injected bilaterally into the hind feet, there was ubiquitous, obvious AchR staining in both 4DL muscles (Fig 3.3).

Consistency of Unlabelled α -BTX Injections (14 days of continuous TTX superfusion and α -BTX injections)

On day 14, none of the muscles in the right hind foot contracted when their nerve supplies were cut *in vitro*. Intracellular recording from one experimental muscle confirmed that the AchRs were functionally silent. Of 30 muscle fibres impaled, none showed evoked synaptic responses to 25 consecutive stimulations of the SN and the LPN (Fig 3.4a, upper trace). In addition, there was no evidence of spontaneous activity in any of the fibres, assessed by the complete absence of MEPPs in 30 second intervals after the nerve stimulations (Fig 3.4a, lower trace). Contralateral control muscles all contracted when their nerve supplies were cut *in vitro*. Intracellular recording from one muscle showed (1) evoked responses to LPN and SN stimulation (Fig 3.4b, upper trace) and (2) MEPPs (Fig 3.4b, lower trace).

Bathing experimental muscles in TRITC- α -BTX on day 14 failed to reveal unblocked AchRs. There was no visible receptor staining anywhere in either of the 2 paralysed muscles (Fig 3.5). In contrast, TRITC- α -BTX revealed extensive AchR staining throughout the contralateral control muscles (Fig 3.5).

Patency of the AchR Block Between Injections (14 days of continuous TTX superfusion and 13 days of α -BTX injections)

In the experimental muscle, the TRITC- α -BTX injection on day 13 did not disclose any AchRs which had previously failed to be blocked with unlabelled α -BTX during the period of paralysis (Fig 3.6). However, the receptors of the contralateral muscle were uniformly stained by the TRITC- α -BTX injection on day 13 (Fig 3.6).

There were also no physiological signs that the unlabelled α -BTX was ineffective between injections: one experimental muscle showed no EMG response to stimulation (Fig 3.7a), yet there was a clear muscle EMG response in a SN crush-only control muscle (Fig 3.7b).

Finally, bath applied TRITC- α -BTX did reveal some AchRs that had not been blocked between the final unlabelled α -BTX injection and the acute experiment. There was some punctate AchR staining at a few endplates in the paralysed muscle, but the staining was confined to one or more small regions, rather than being uniform throughout the receptor plaque (Fig 3.8a). Overall, the level of punctate staining was very low: only 14 fibres in the muscle (approximately 1.4%) showed any traces of rhodamine dye. In contrast, receptor plaques in the contralateral muscle appeared uniformly stained, and there was evidence of uniform receptor staining throughout the muscle (Fig 3.8b).

3.5 Effects of SN Denervation on LPN Sprouting

Three days after crush, there was no muscle response to stimulating the SN, in any of the six nerve-muscle preparations studied *in vitro*. One of the muscles contained no partially innervated or unoccupied endplates; all the receptor patches examined in the muscle were 100% occupied by LPN terminals (n=105 fibres; examples not shown). This muscle was discarded from further analysis and is not included in the description of the results that follow.

In all of the remaining muscles in the group (n=5), the majority of the fibres consisted of receptor patches that were fully occupied by the uninjured LPN terminals (Fig 3.9a); the proportion of fully occupied endplates in the muscles was $78 \pm 5.0\%$.

Partially occupied endplates comprised $15.4 \pm 3.6\%$ of the fibres examined, and were characterised by small regions of the receptor plaque that were not overlaid by nerve terminal staining (Fig 3.9b). The fraction of the endplate left vacant was very small at all the partially occupied fibres: there were no endplates where more than 35% of the receptor plaque was unoccupied.

LPN terminal sprouting was visible, but not excessive: only $10.4 \pm 1.9\%$ of endplates received ($2.8 \pm 0.9\%$) or projected ($7.6 \pm 1.7\%$) a nerve sprout. The majority of endplates which projected a nerve sprout were partially occupied ($71.3 \pm 5.6\%$; Fig 3.10a) and the remainder of sprouts were extended from fully occupied endplates (Fig 3.10b). All of the endplates receiving a sprout were vacant (Fig 3.10c).

Finally, a minority of fibres in all the muscles was completely vacant ($6.6 \pm 1.7\%$; Fig 3.11).

3.6 Pattern Of 4DL Innervation 14 Days After SN Crush

Fourteen days after SN crush there was no *in vitro* muscle response to SN stimulation in any of the muscles in the group (n=13). The majority of receptor patches were fully occupied by LPN terminals (1430/1483 fibres studied in total

from the 13 muscles; Fig 3.12a). The proportion of fully occupied endplates was significantly greater at 14 days than at 3 days after SN crush ($P<0.01$).

Partially occupied endplates were detected in 11 of the 13 muscles, comprising $3.4 \pm 0.7\%$ of all the fibres analysed in the group. The mean percentage of the receptor plaques not covered by nerve terminal staining 14 days after SN crush ($88.9 \pm 0.8\%$; Fig 3.12b) was comparable to that seen 3 days after crush (described above): there were no endplates where more than 20% of the receptor plaque was unoccupied. In fact, there was no significant difference between the size of unoccupied patches 3 or 14 days after crush ($P>0.05$). The correlation between total endplate size and the fraction of the endplates that were occupied was not significantly different from zero (Fig 3.13; $r=-0.01$).

Two unoccupied endplates were observed in one of the muscles, indicated by the absence of any nerve terminal and axonal staining over the area of receptor staining (Fig 3.14). Adjacent endplates were stained with neurofilament, SV2 and TRITC- α -BTX. The total number of endplates examined in the series of experiments was 1483 and so the proportion of unoccupied endplates was 0.1% overall.

Finally, the incidence of nerve sprouting in the muscles was very low: only $4.1 \pm 0.6\%$ of endplates received ($1.8 \pm 0.4\%$) or projected ($2.3 \pm 0.3\%$) a nerve sprout (Fig 3.15). There was significantly less sprouting between endplates 14 days vs. 3 days after SN crush ($P<0.001$). Neither of the unoccupied endplates described above

received a nerve sprout, even though there were several fully innervated endplates in their close vicinity (see Fig 3.14).

3.7 Effects of Activity Block on Synapse Elimination

3.7.1 Patterns of Innervation

Muscles which had been paralysed with TTX or α -BTX for two weeks showed elevated levels of polyneuronal innervation, compared to contralateral controls, however, the differences were not quite significant between groups (one way ANOVA, $P=0.059$; Fig 3.16). The highest level of polyneuronal innervation was found in the TTX/ α -BTX blocked muscles ($13.1 \pm 4.5\%$ of fibres), followed by the TTX blocked muscles ($10.1 \pm 2.3\%$; $P>0.05$). The lowest levels were found in contralateral controls ($2.3 \pm 0.8\%$).

The majority of fibres in all 3 groups were mononeuronally innervated by the LPN (Figs 3.16 & 3.17a). Levels of LPN mononeuronally innervated junctions were highest in the controls ($96 \pm 0.4\%$ of fibres), followed by the TTX blocked muscles ($85 \pm 3.4\%$) and TTX/ α -BTX blocked muscles ($84.1 \pm 5.2\%$). There were no significant differences between the percentage of LPN mononeuronally innervated fibres in the 3 conditions (one way ANOVA, $P>0.05$).

Finally, there were SN mononeuronally innervated endplates in all 3 groups (Fig 3.17b). Like the other patterns of innervation, the levels of SN mononeuronally innervated endplates were not significantly different between conditions (one way ANOVA, $P>0.05$; Fig 3.16). In the control muscles, only $1.9 \pm 0.4\%$ of fibres were occupied exclusively by SN axons. This was not significantly less than the

proportion in either the TTX blocked muscles ($4.9 \pm 1.7\%$) or TTX/ α -BTX blocked muscles ($4.0 \pm 1.0\%$), when post-hoc tests were carried out at the 5% significance levels. There was also no significant difference between the amount of SN mononeuronally innervated endplates in the activity blocked muscles.

Some of the SN mononeuronally innervated endplates from the TTX blocked muscles were relocated after immunocytochemical/TRITC- α -BTX staining. The morphology revealed by the styryl dyes was confirmed: in all junctions, there were no synaptic boutons stained with neurofilament/SV2 that had not been previously labelled by FM1-43 (Fig 3.18).

3.7.2 Polyneuronal Innervation

There were 3 patterns of innervation at the polyneuronal endplates, in all 3 groups of muscles. Sometimes the LPN innervated the majority of the junction (Fig 3.19a), sometimes the SN was the major nerve supply (Fig 3.19b), and sometimes the two nerves occupied (approximately) equivalent amounts of synaptic area (Fig 3.19c).

In the control muscles, an analysis of the fractional occupancies of the convergent axons revealed that the SN was larger than the LPN at 13 out of 17 endplates, and contributed, on average, $63.5 \pm 5.2\%$ of total endplate size (Fig 3.20). There was a significant difference between the sizes of SN ($95.7 \pm 4.1 \mu\text{m}^2$) and LPN ($53.5 \pm 8.4 \mu\text{m}^2$) inputs in the group ($P < 0.05$; paired Wilcoxon t test).

In the TTX block group, the SN was larger than the LPN at 29 of the 41 endplates detected. The average size of SN inputs was $107.2 (\pm 9.3 \mu\text{m}^2)$, which was significantly larger than the size of the LPN inputs ($56 \pm 6.5 \mu\text{m}^2$; $P < 0.001$, paired Wilcoxon t test; Fig 3.20). The average SN contribution to total endplate size was $65 \pm 3.9\%$. Some of the polyneuronally innervated endplates from the TTX blocked muscles were relocated after immunocytochemical/TRITC- α -BTX staining. The morphology revealed by the styryl dyes was confirmed: in all junctions, there were no synaptic boutons stained with neurofilament/SV2 that had not been previously labelled by FM1-43 or RH414 (Fig 3.21).

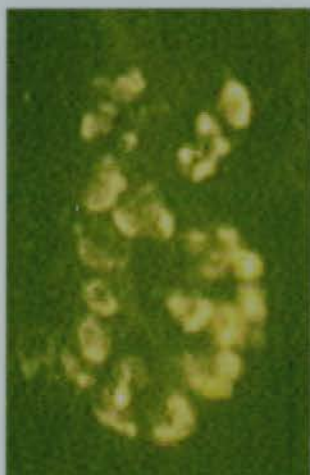
An analysis of the polyneuronally innervated endplates in the TTX/ α -BTX muscles revealed that the SN was the larger of the two inputs at 41 out of 76 endplates. On average, the SN occupied $52 (\pm 2.8) \%$ of total endplate area. However, there was no significant difference between the sizes of SN and LPN inputs (paired t test; SN size was $90.7 \pm 6.8 \mu\text{m}^2$; LPN size was $81.3 \pm 6.2 \mu\text{m}^2$; Fig 3.20).

There were also no significant differences between the sizes of SN inputs at the polyneuronally innervated junctions in the 3 groups (one way ANOVA, $P > 0.05$; Fig 3.20). When differences between the sizes of the LPN inputs were analysed there was a significant difference between the 3 groups (one way ANOVA, $P < 0.05$). However, post-hoc tests revealed that the only significant difference was between the size of the LPN inputs in the TTX and TTX/ α -BTX groups ($P < 0.05$; Fig 3.20).

Finally, there were no significant correlations between total endplate size and the fractional occupancy of the SN or the LPN, in all 3 groups (Fig 3.22). There were instances where the SN comprised the minor input, was equivalent in size to the LPN, or formed the dominant input, irrespective of the total size of the endplate.

Fig 3.2 Examples of the typical morphology of endplates in 4DL muscles 3 days after sham implants. In all the junctions examined, there was continuous nerve terminal staining (FM1-43; left hand side panels) over the receptor patches (TRITC- α -BTX; right hand side panels). Top and bottom junctions are from two separate animals. Scale bar is 20 μ m.

FM1-43



TRITC- α -BTX

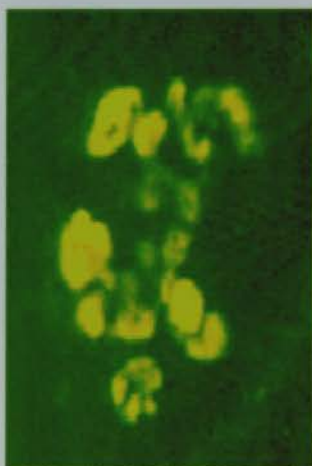
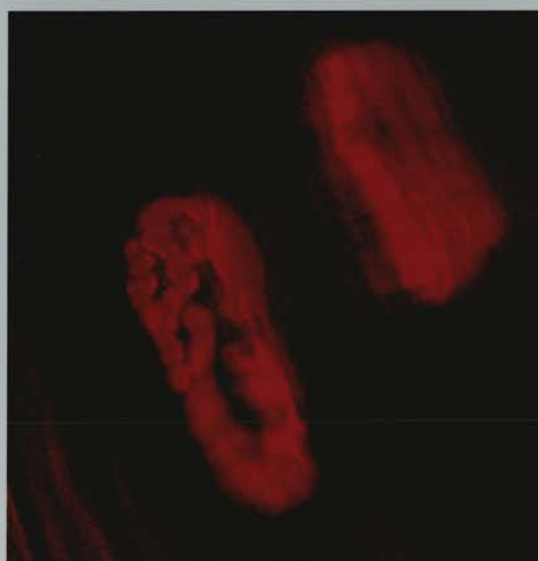
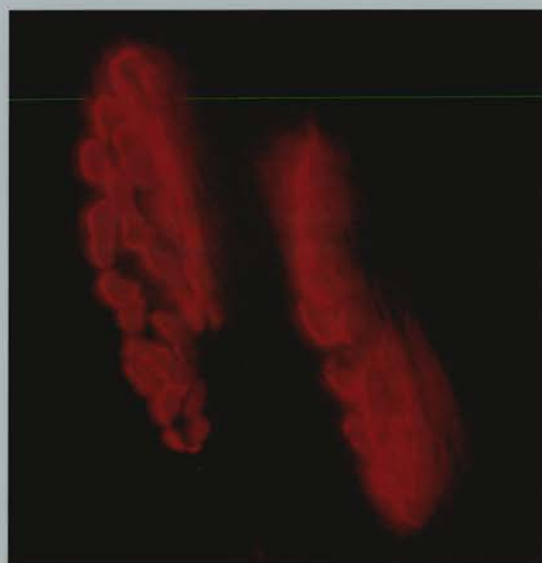


Fig 3.3 TRITC- α -BTX injections successfully labelled 4DL AchRs. A single injection was given, bilaterally, and nerve-muscle preparations were made and viewed with fluorescence microscopy 24 hours later. Top and bottom panels are examples from the right and left 4DL muscles, respectively. Scale bar is 20 μ m.



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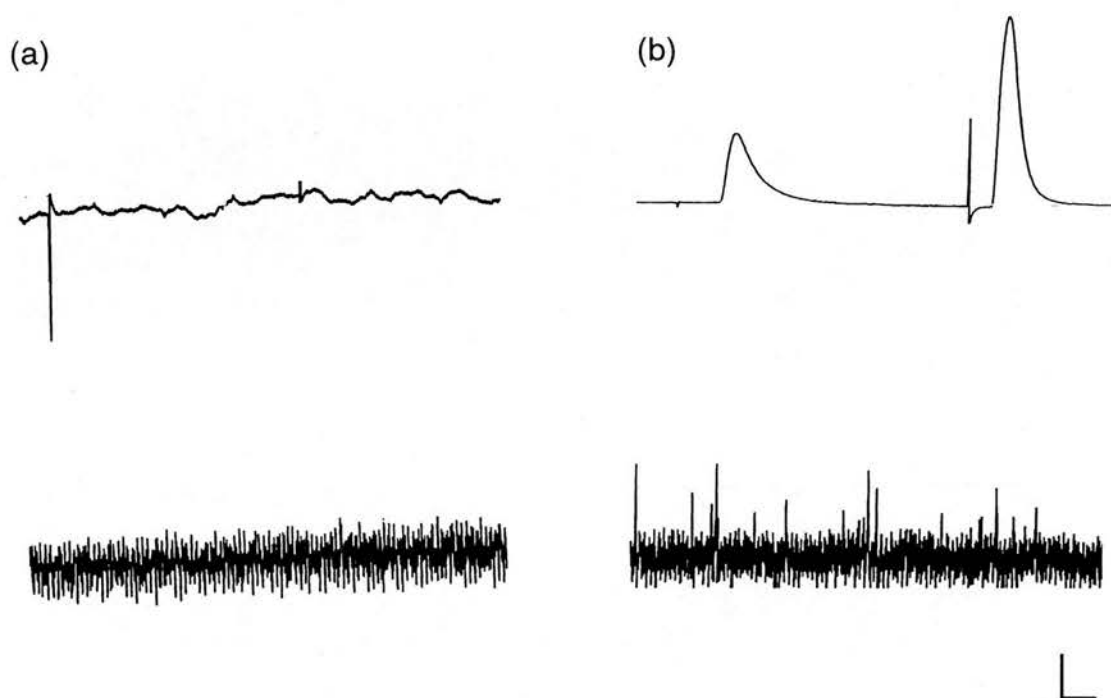


Fig 3.4 Intracellular recording demonstrated that unlabelled α -BTX consistently labelled AchRs in 4DL muscles. MEPPs and evoked responses to LPN/SN stimulation were recorded from a TTX/ α -BTX blocked 4DL muscle (left hand traces) and the contralateral control (right hand traces). In the upper traces, the timing of LPN and SN stimulation, respectively, can be determined by the positions of the stimulus artifacts. In the lower traces, MEPPs appear as peaks above the baseline noise level. (a): none of the fibres in the blocked muscles showed EPPs in response to LPN or SN stimulation (upper trace) or MEPPs (lower trace). (b): EPPs and MEPPs were recorded in all fibres sampled in the contralateral control muscle. The example shown in the upper trace is a polyneuronally innervated fibre. *Scale bars:* (a): upper trace: 0.2msec/20mV. (b): upper trace: 0.2msec/5mV. Lower traces: 2sec/0.5mV

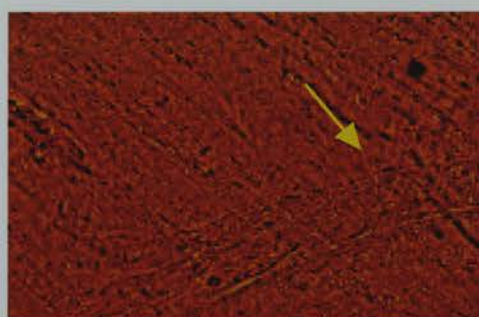
Fig 3.5 4DL AchRs were reliably blocked at the time of the acute experiment, following 14 days of daily unlabelled α -BTX injections to the right hind foot. On day 14, nerve-muscle preparations from a TTX/ α -BTX blocked muscle and the contralateral control 4DL were made and bathed at the same time in TRITC- α -BTX. Rhodamine fluorescent staining from the contralateral control (left hand panel) was visible throughout the muscle; there was no apparent staining in the injected muscle (right hand panel), but note the background staining, indicating non-specific binding of TRITC- α -BTX. Scale bar is 40 μ m.



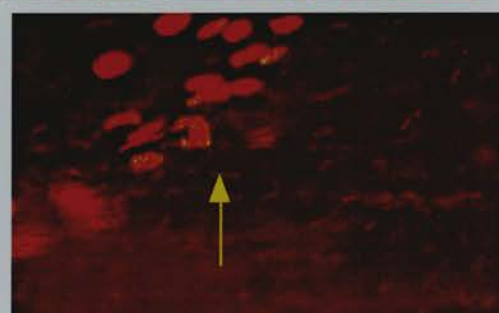
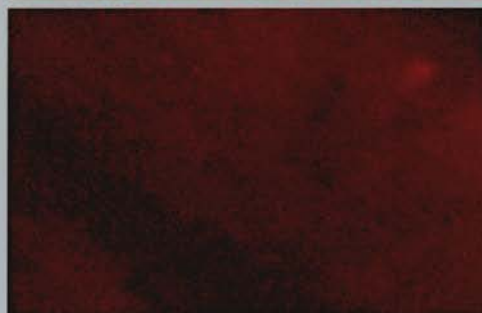
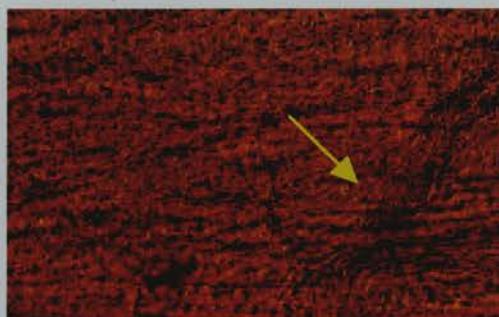
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Fig 3.6 AchRs were consistently blocked between daily unlabelled- α -BTX injections. A reinnervated 4DL muscle received TTX block and 12 days of unlabelled- α -BTX injections. On day 13 TRITC- α -BTX was injected over the 4DL (TTX/ α -BTX Blocked; left hand panels). The contralateral control was reinnervated only, and injected on day 13 with TRITC- α -BTX (right hand panels). The animal was sacrificed 24 hours after the bilateral TRITC- α -BTX injection and the muscles were dissected. The top panels show the appearance of the muscles under transmitted light; the nerve trunks are visible running from the lower left to upper right of the images, indicated by the arrows. Neuromuscular junctions are highly concentrated in the region of the nerve trunk. The bottom panels show the appearance of the muscles under fluorescent light, revealing the location of any TRITC- α -BTX staining. There was no obvious AchR staining in the activity-blocked muscle, but the background fluorescence indicates non-specific binding of TRITC- α -BTX. There was ubiquitous staining throughout the contralateral control 4DL, indicated by the arrow. Scale bar is 80 μ m.

TTX / α -BTX Blocked



Contralateral Control



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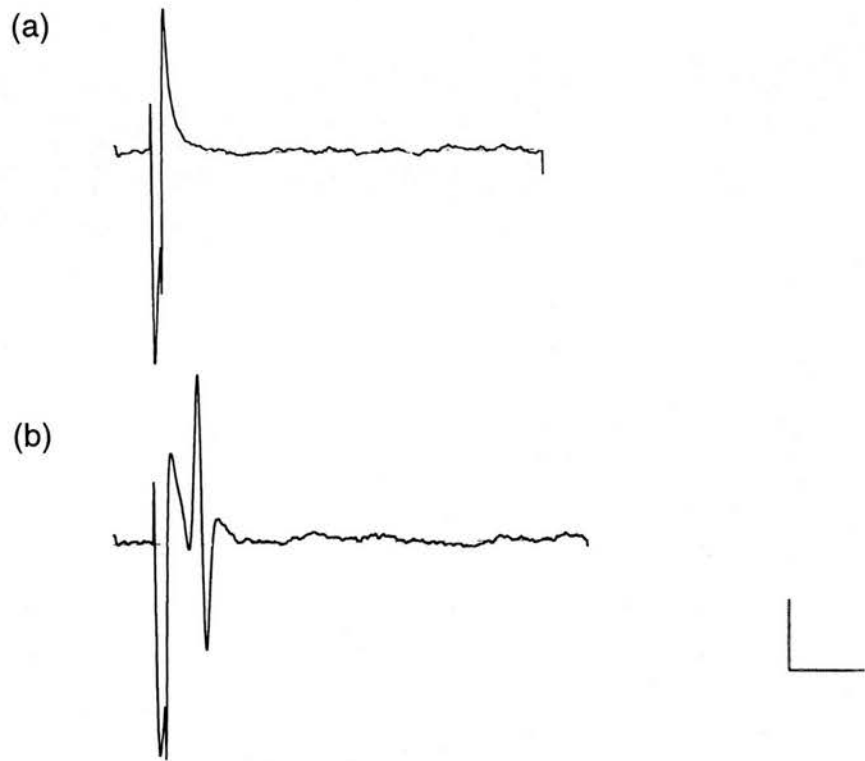


Fig 3.7 Extracellular recordings from the 4DL confirm that unlabelled α -BTX blocked AchRs throughout the muscle. The 4DL muscle nerve (containing all LPN and SN axons) was stimulated supramaximally. (a): Following a short delay and the appearance of the stimulus artifact at the beginning of the trace, it was apparent that there was no muscle response to stimulation in the α -BTX blocked muscle. (b): Following a short delay and the appearance of the stimulus artifact at the beginning of the trace, a large compound action potential was recorded in the contralateral control 4DL. Time base is 0.5msec/20mV.

Fig. 3.8 Bath applied TRITC- α -BTX revealed the location of unblocked AchRs, 24 hours after the last unlabelled α -BTX injection. Top panel: a reinnervated TTX blocked 4DL was given unlabelled α -BTX injections for 13 days. On day 14, the muscle was dissected and bathed in TRITC- α -BTX. Punctate staining was visible where the rhodamine α -BTX partially labelled the receptor plaques. Bottom panel: the reinnervated contralateral control 4DL was stained with TRITC- α -BTX at the same time as the activity-blocked muscle (on day 14). There was robust, uniform AchR staining, where the TRITC- α -BTX saturated the receptor plaques. Scale bar is 20 μ m.

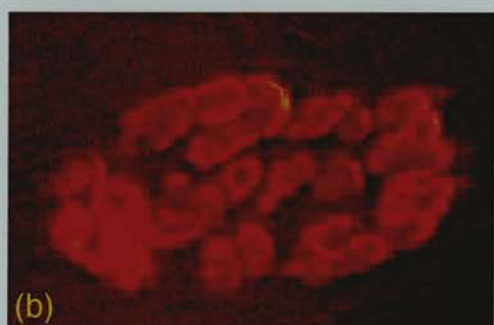
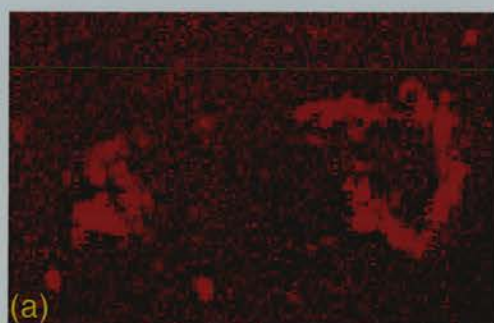


Fig. 3.9 The LPN rapidly sprouts to innervate endplates left denervated by SN crush in the 4DL. Left hand panels show nerve terminal boutons stained with FM1-43; right hand panels show the configuration of receptors at the endplates, stained with TRITC- α -BTX. Three days after SN crush, the majority of muscle fibres were fully occupied by intact LPN axons (a), indicated by a 100% overlap between the area of the nerve terminal and AchR staining. At the partially occupied endplates, only very small portions of the receptor plaque showed no overlying nerve terminal staining (b). The yellow arrows indicate non-innervated regions of the endplate. Scale bar is 10 μ m.

FM1-43

TRITC- α -BTX

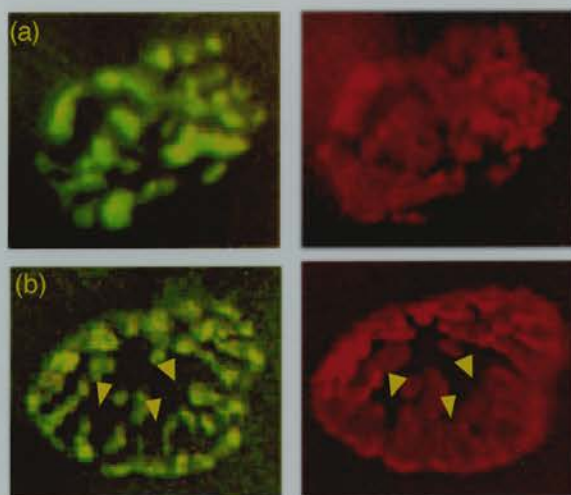
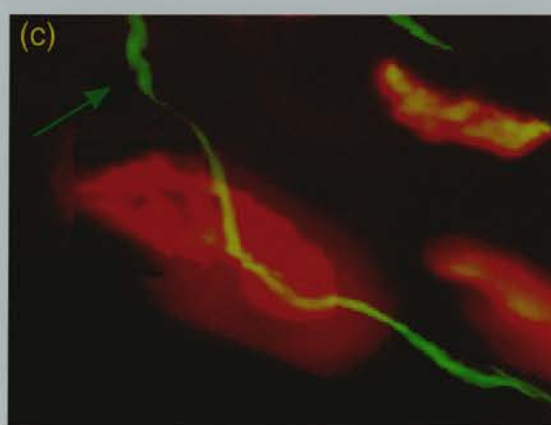
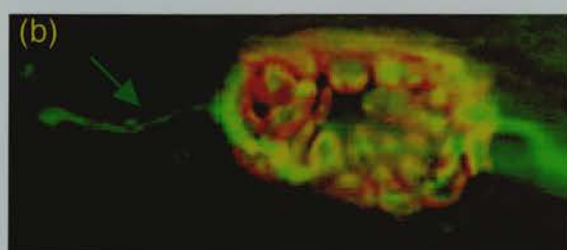
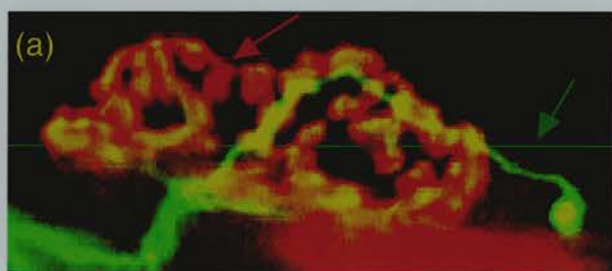
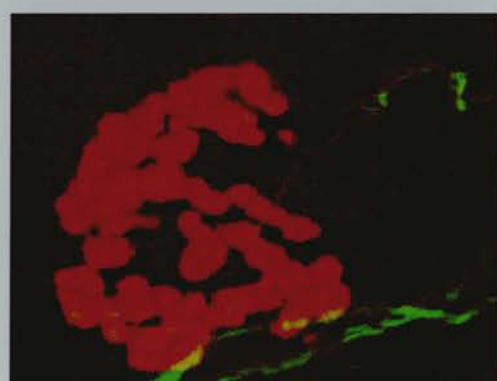
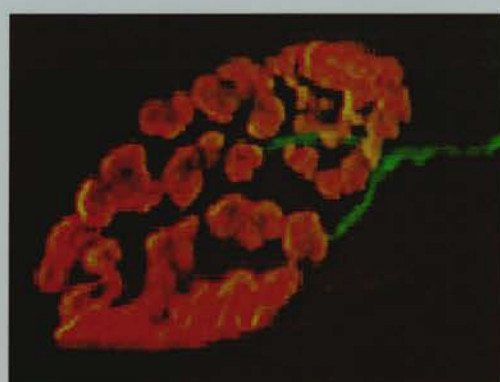


Fig 3.10 There were 3 patterns of LPN terminal sprouting, 3 days after SN crush in 4DL muscles. AchRs were labelled with TRITC- α -BTX, and nf/SV2 were immunolabelled with FITC antibodies, so nerve terminals overlying AchRs and areas where axons travel across the endplates appear yellow. Immunocytochemical staining revealed that sprouts extended from either partially (a) or fully (b) occupied endplates towards, or making contact with denervated endplates (c). The red arrow in (a) shows a small cluster of AchRs without innervation. Green arrows indicate nerve sprouts; the unmarked axons at the endplates belong to the parent, collateral axons. Scale bar is 20 μ m.



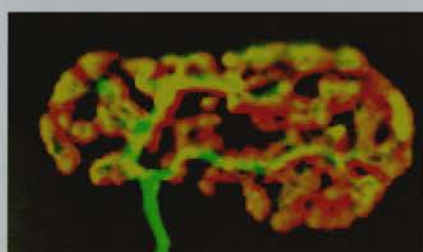
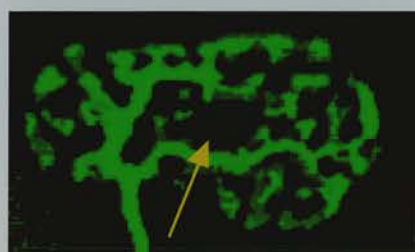
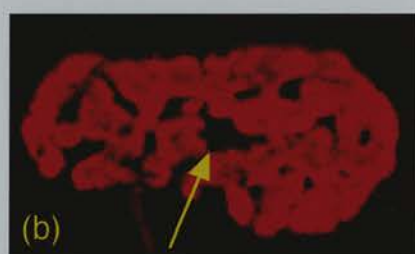
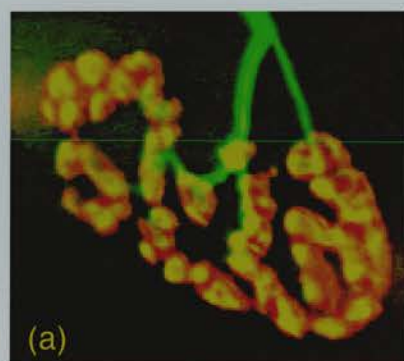
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Fig 3.11 Few 4DL endplates were without innervation 3 days after SN crush. The top and bottom panels are examples of 100% vacant AchR plaques from 2 different muscles. In each case, note the presence of the innervating axons at the right of the endplates. Scale bar is 20 μ m.



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Fig 3.12 The LPN successfully innervated 4DL muscles 14 days after SN crush. The majority of endplates were fully innervated (a), indicated by the 100% overlap between FITC-nf/SV2 and TRITC- α -BTX staining. Partially occupied endplates were more difficult to find, and were characterised by very small non-innervated regions of the AchR plaque (b). In the example given in (b), the top panel shows AchR staining alone and the middle panel shows the presynaptic staining alone. The bottom panel shows the pre- and postsynaptic structure, and reveals the degree of overlap. The yellow arrow in the first 2 panels indicates the region of non-innervation; this area appears red, instead of yellow, in the bottom panel. Scale bar is 20 μ m.



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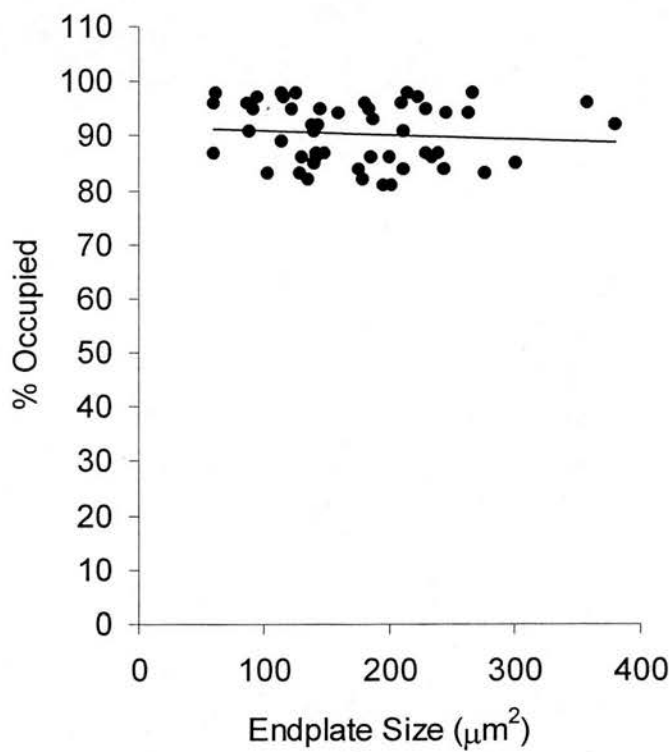
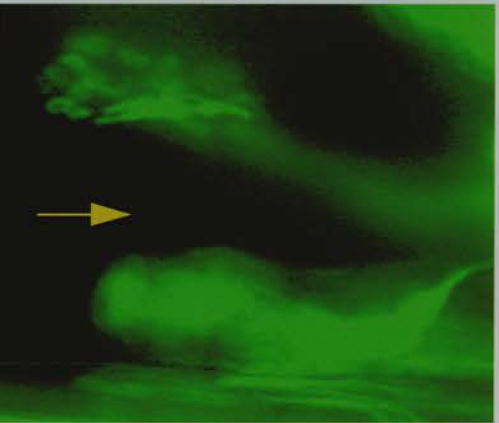
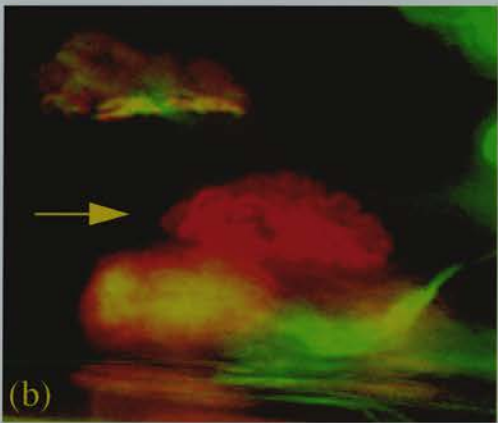
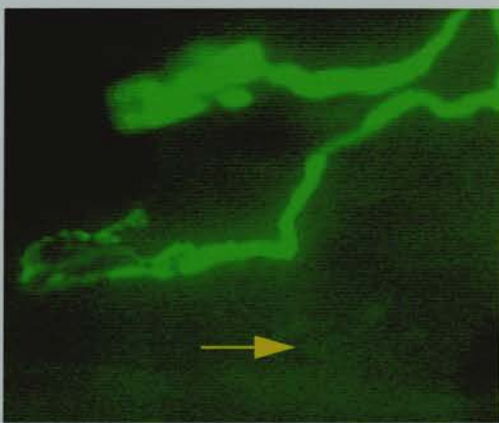
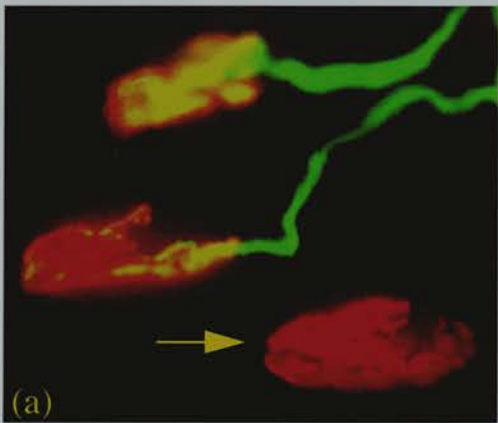


Fig 3.13 Small 4DL endplates were no more likely to be partially innervated than large 4DL endplates. The endplate size (determined from the area of AchR staining) of all 51 partially occupied endplates was plotted against the amount of the endplate that was innervated (% of the AchR plaque covered by nerve terminal staining). The slope of the regression through the data is -0.01.

Fig 3.14 Two 4DL endplates failed to attract LPN innervation 14 days after SN crush. Presynaptic structures were immunolabelled with FITC antibodies to nf and SV2; AchRs were stained with TRITC- α -BTX (left hand panels). Right hand panels show the presynaptic staining alone. Arrows indicate the location of AchR-positive staining and nf/SV2 negative staining. Both vacant endplates were found in the same muscle. In both cases, adjacent endplates were within a distance of 30 μ m or less, and were positively stained for AchRs, axons, and nerve terminals. Scale bar is 20 μ m.

FITC anti NF/SV2/TRITC α -BTX

FITC anti NF/SV2



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Fig 3.15 Nerve sprouting was detected in 4DL muscles 14 days after SN crush. Top and bottom panels are examples of 100% innervated endplates from 2 different muscles, stained immunocytochemically. The yellow arrows indicate terminal sprouts; the unmarked axons are derived from parent, collateral axons. Scale bar is 30 μ m.

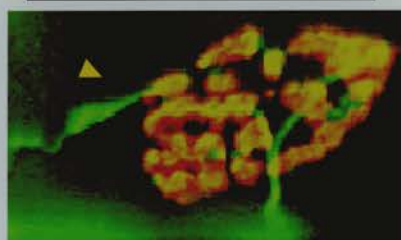


Fig 3.16 Patterns of innervation in 4DLs varied 28 days after SN crush, depending on the level of activity in the muscles immediately following reinnervation. For each graph, the mean percentage of muscle fibres that were LPN mononeuronally innervated (designated LPN), polynuronally innervated (poly), or SN mononeuronally innervated (SN) in each group of muscles are given. Error bars are \pm s.e.m. The bracketed numbers refer to the number of muscles in each group/number of endplates analysed in total. A one-way ANOVA and Dunn's post-hoc tests for each pattern of innervation were performed. There were no significant differences between the levels of LPN, SN or polynuronally innervated endplates between any of the groups.

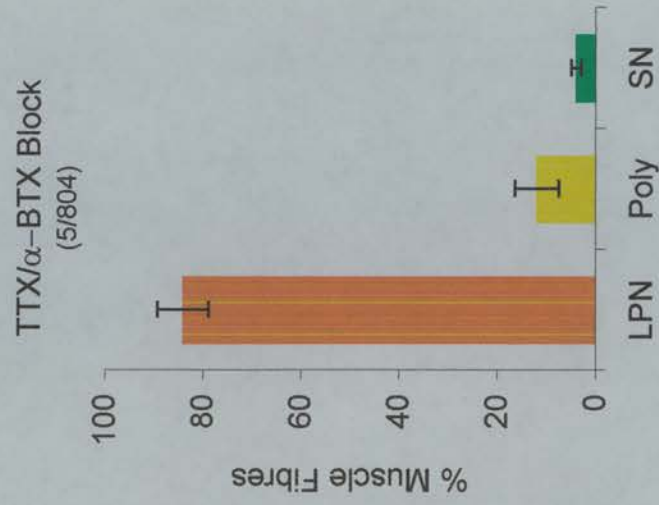
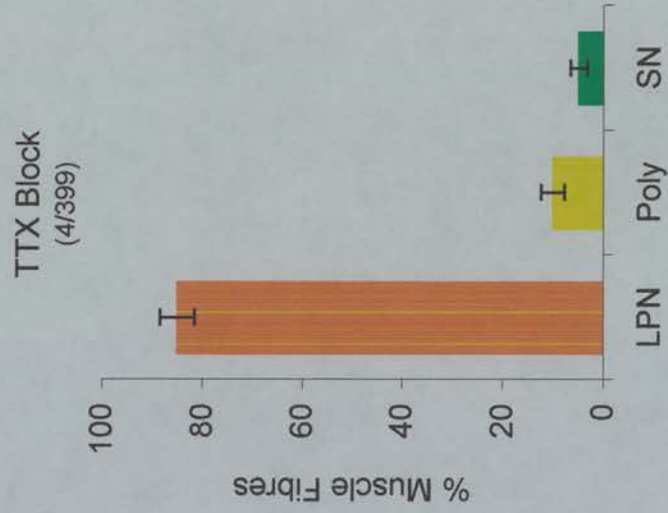
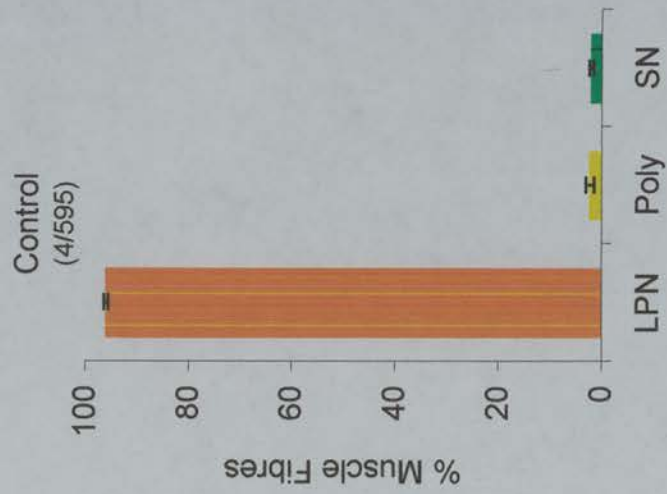
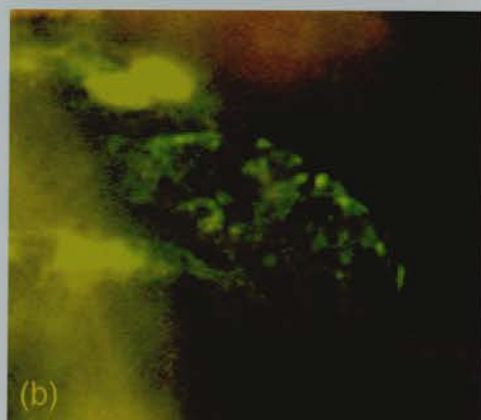
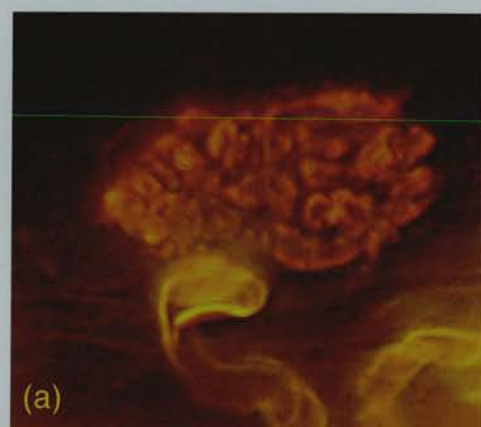
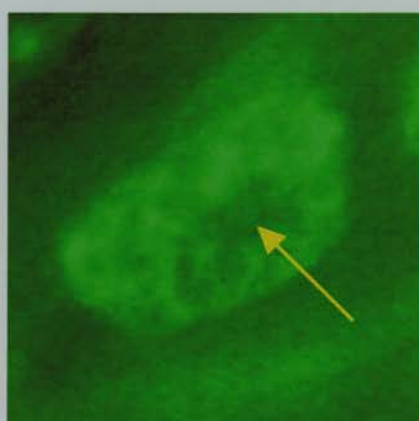
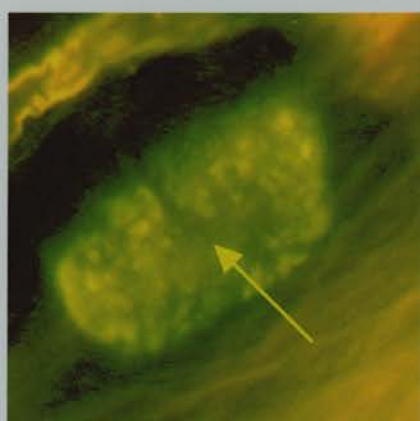


Fig 3.17 There were LPN and SN mononeuronally innervated endplates in all the 4DL muscles, in all 3 activity conditions. LPN mononeuronal innervation was indicated by the presence of RH414-only stained boutons at the endplate (a). The example is from a muscle in the TTX group. SN mononeuronally innervated endplates were stained exclusively by FM1-43 (b). The example is from a muscle in the TTX/ α -BTX group. Scale bar is 20 μ m.



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Fig 3.18 FM1-43 did label all the inputs to SN mononeuronally innervated endplates in 4DL muscles. The left hand panel shows an example of nerve terminal staining with FM1-43. The yellow arrow indicates a region where there were no synaptic boutons. The right hand panel shows the same endplate, relocated after fixing the muscle and immunocytochemical staining. The yellow arrow indicates the same region, demonstrating that there were no boutons that had failed to be stained by the vital dyes. The example is from a muscle in the TTX group. Scale bar is 20 μ m.



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Fig 3.19 Polyneuronally innervated 4DL endplates were indicated by selective labelling of convergent axons with FM1-43 (green) and RH414 (orange). There were 3 patterns of polyneuronally innervated endplates 28 days after SN crush. Sometimes the LPN provided the majority of the endplate innervation (a), sometimes the SN was the major nerve supply and the LPN provided the minority of innervation (b), and some endplates were innervated (approximately) equally by the LPN and SN (c). Scale bar is 20 μm .

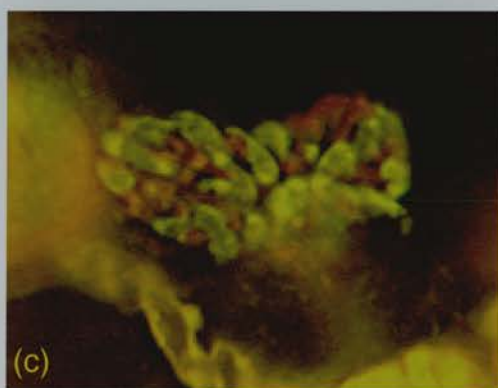


Fig 3.20 The fractional occupancies of the LPN and SN at the polyneuronally innervated 4DL endplates. For each experimental condition, the mean size of the SN and LPN inputs to the polyneuronally innervated junctions are given. Error bars are \pm s.e.m. The bracketed numbers refer to the number of muscles in each group/total number of polyneuronally innervated endplates detected in the group of muscles. A one-way ANOVA and Dunn's post-hoc tests for each pattern of innervation were performed. * indicates a significant difference between the mean size of the LPN and SN within the group. ** indicate a significant difference between the mean size of LPN or SN inputs between groups. The only significant between-group difference was for the size of LPN inputs in the TTX blocked muscles and the TTX/ α -BTX muscles.

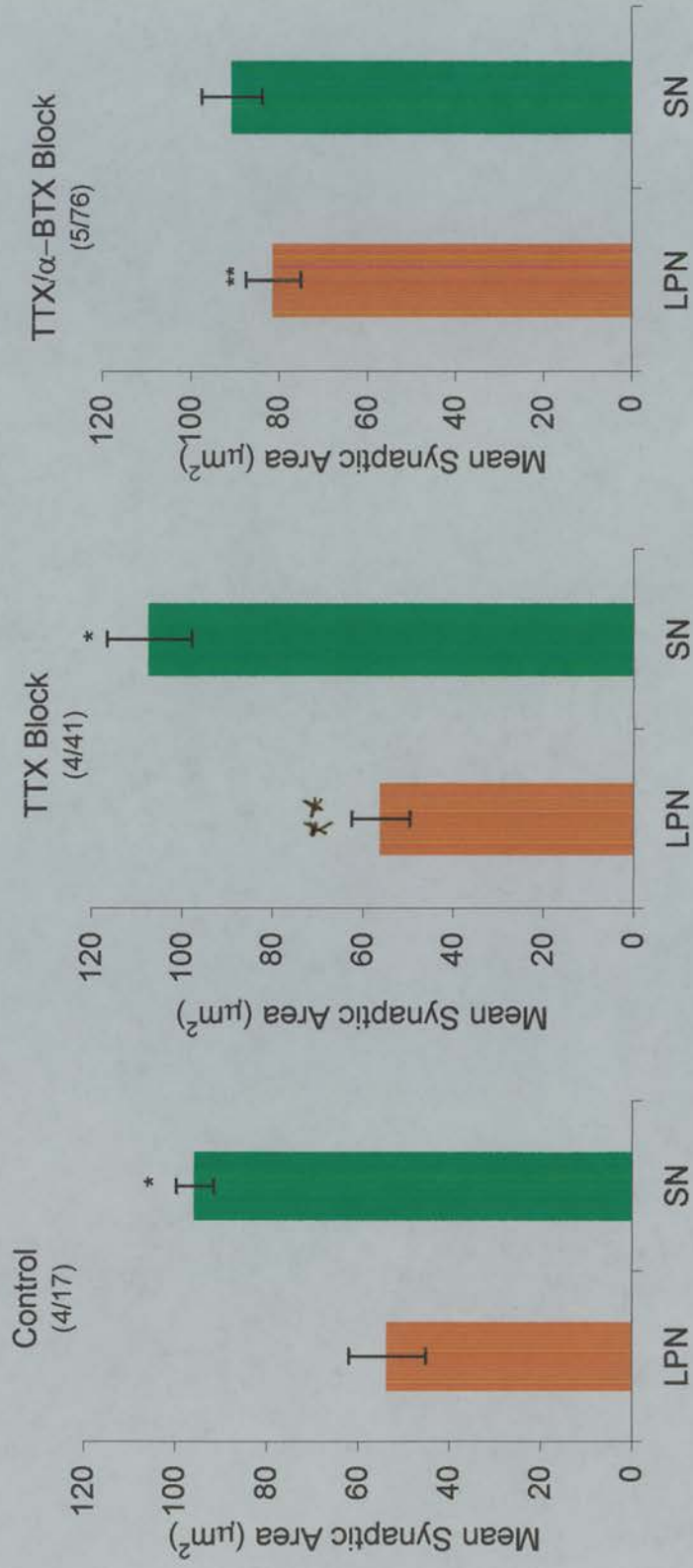
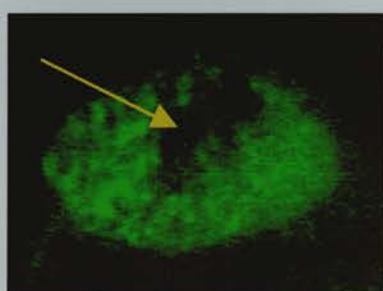
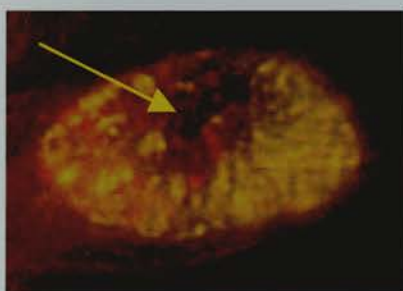
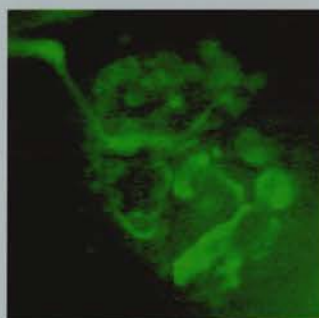
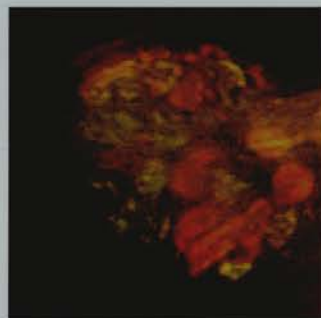
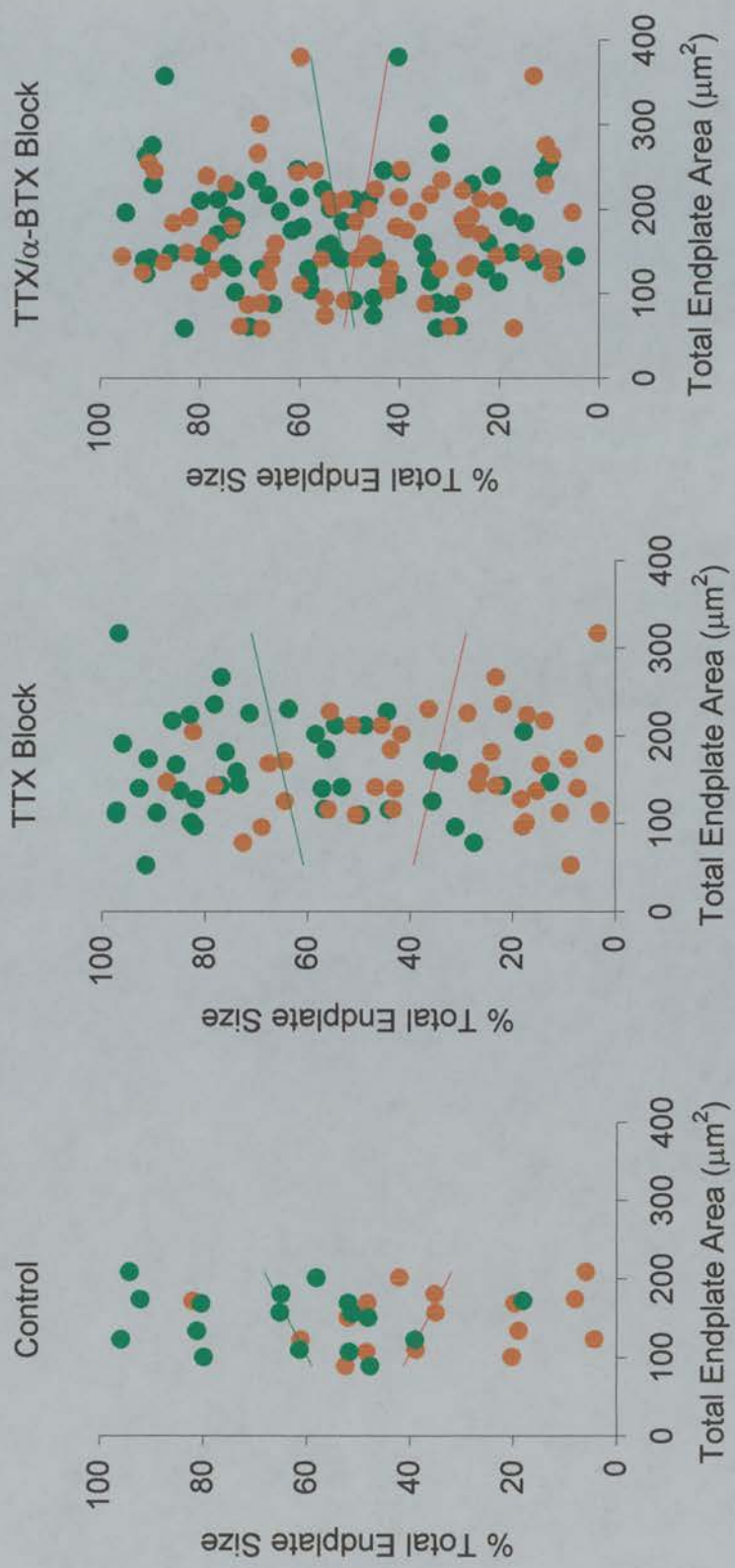


Fig 3.21 Immunocytochemical staining confirmed that 4DL endplates were polyneuronally innervated and that all the boutons of both convergent inputs had all been labelled with FM1-43 and RH414. The left hand panels show 2 polyneuronally innervated endplates, stained with the styryl dyes. The right hand panels are the same junctions, relocated after fixing and immunolabelling. In the bottom example, the yellow arrows indicate a region of the endplate that was without innervation. No boutons were revealed with immunocytochemical staining that had not been previously identified with FM1-43 or RH414. Scale bar is 20 μ m.



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Fig 3.22 The distribution of SN/LPN convergent inputs. In all 3 conditions, the total size (μm^2) of every polynuronally innervated endplate was plotted against the contribution (in %) to total endplate size made by each of the convergent inputs (thus, each endplate is plotted twice, once against the SN % occupancy and once against the LPN % occupancy). Orange circles are LPN data, and green circles are data from the SN. In all 3 conditions, there were instances where the SN was the smaller of the 2 inputs (contributing less than 50% of the total amount of innervation at the endplate), equal in size to the LPN, or was the larger of the 2 inputs (providing greater than 50% of total innervation). However, in the TTX blocked muscles, the SN was the larger input at most of the polynuronally innervated junctions. There were no significant correlations between endplate size and the % occupancy by the SN or the LPN in any of the 3 conditions (*control muscles*: Pearson's $r=0.1$, SN data set; $r=-0.1$, LPN data set. *TTX-blocked muscles*: $r=0.1$, SN data set; $r=-0.1$, LPN data set. *TTX/ α -BTX blocked muscles*: $r=0.07$, SN data set, $r=-0.07$, LPN data set; significance was tested at 5%, for difference from a Pearson correlation of 0.00).



Discussion

In the experiments described here, the influence of activity on the ability of axonal inputs to occupy endplates was tested. TTX was used alone or in conjunction with α -BTX to abolish activity at 4DL neuromuscular junctions, so that inactive synapses were forced to compete against one another for the innervation of endplates on inactive muscle fibres. There are two points of general interest established by the results. The first is that synapse elimination proceeds at some endplates when activation of the AchRs – by both evoked and spontaneous release – is blocked at the time of the reinnervation of skeletal muscle. Second, Ach-AchR activation is not a necessary condition for synapse elimination, a result which suggests a presynaptic locus of control for activity-independent mechanisms of synaptic plasticity.

In interpreting these observations, a variety of potential sources of error have been considered.

3.8 Nerve Damage Caused By Sciatic Cuffs and/or the Implanting Technique

A common and useful experimental approach to blocking nerve conduction has been to apply a cuff containing a slow-release chemical to peripheral nerves. The early attempts used local anaesthetics (Robert & Oester, 1970; Lømo & Rosenthal, 1972). However, later developments showed that the major part of the blocking effect was due to mechanical compression of the nerve (Cangiano et al., 1977) and controlled nerve compression can produce long-lasting conduction blocks due to demyelination (Ochoa et al, 1972). Thus, using some paralysis paradigms, it may be difficult to distinguish between the effects of impulse block and inactivity on the one hand, and

nerve degeneration on the other. The present technique offers the advantage that cuffs were loosely fitted around the sciatic, minimising the chances of nerve damage and degeneration. Nevertheless, if some LPN axons were damaged in the process of inserting a cuff, nerve terminals belonging to the injured axons should have withdrawn from the muscle fibres within 24 hours (Waller, 1852). If so, the presence of SN mononeuronally innervated endplates after paralysis might not reflect competition, but simply that regenerating SN axons had occupied vacant endplates. However, control experiments suggest it is unlikely that this was the case: the sham-implanted controls showed no clinical signs of limb paralysis and no vacant endplates three days after implant (see **Fig 3.2**). Because there were SN-only innervated junctions in all the muscles in the TTX and TTX/ α -BTX groups, it would have to be argued that the implanting technique damaged LPN axons every time the procedure was performed. There is no reason to expect that the same damage would not have been caused in the control experiments. Thus, it is concluded that the effects of paralysis described here were not the result of some mechanical effect produced by the insertion of the cuffs; SN innervated endplates can be attributed to successful competition for endplate occupancy, and not to vacant endplates caused by LPN damage and degeneration.

3.9 The Presence Of Vacant and Partially Occupied Endplates When SN Axons Returned To 4DL Muscles (14 Days After SN Crush)

A related consideration is that some muscle fibres were unoccupied by LPN terminals when regenerating SN axons returned to the 4DLs. Ribchester (1993) noted this potential source of error in his discussion, speculating that it was possible some fibres denervated by the SN crush failed to attract sprouts/innervation from

LPN axons. He dismissed the possibility as unlikely, in part because it failed to explain why there should be more non-sprout-attracting fibres in TTX blocked muscles than controls. While this argument is reasonable, a closer inspection of his data reveals that the muscles in the TTX blocked group had, on average, more SN motor units than controls. After SN crush, 4DL muscles with many SN motor units would have been left with more denervated fibres than muscles with few SN motor units. If the LPN was unable to restore complete innervation to the larger numbers of fibres left denervated by SN crush in the TTX group, the increased levels of observed SN reinnervation could reflect sampling error, and not activity independent synapse elimination. This criticism is reinforced when it is considered that the number of muscles included in Ribchester's study was small ($n=5$). However, it has been demonstrated that only three motor units are required to provide full compensatory innervation in the 4DL following partial denervation (Ribchester, 1988). Since the 4DL is supplied by 10-12 motor units, it is probable that there were enough LPN axons to recover innervation of vacant endplates following SN crush in the 1993 study. Nonetheless, Ribchester's arguments would be strengthened by a demonstration that all the endplates denervated by SN crush were at least partially occupied at the time of TTX paralysis, regardless of how many SN axons supplied the muscle.

The results of the present study confirm and extend Ribchester's earlier results. Of the 1483 muscle fibres analysed 14 days after SN crush, only 2 were vacant (see **Fig 3.14**). While this finding would tend to support the idea that the LPN does not always successfully reinnervate injury-induced denervated 4DL fibres, the vacant

endplates amount to just 0.1% of muscle fibres. If this reflects the proportion of denervated fibres that fail to become innervated by sprouts, then only 0.1% of subsequent SN mononeuronally innervated endplates (after activity block) may be accounted for by SN axons growing back to unoccupied fibres. However, in the TTX and TTX/ α -BTX blocked muscles, 4.9% ($\pm 1.7\%$) and 4.0% ($\pm 1.0\%$) of the endplates were SN mononeuronally innervated, respectively. Even in the contralateral control muscles, the proportion of SN only innervated endplates – 1.9% ($\pm 0.4\%$) – was substantially higher than would be expected, based on the small number of vacant endplates seen 14 days after SN crush.

The presence of polyneuronally innervated junctions after activity block also supports the idea that regenerating SN inputs eliminated intact LPN inputs. Fourteen days after SN crush, there were very few partially occupied endplates in the muscles ($3.4 \pm 0.7\%$). However, in the TTX, and TTX/ α -BTX blocked groups, the proportions of polyneuronally innervated junctions were $10.1 \pm 2.3\%$ and $11.9 \pm 4.5\%$, respectively. Since there were more polyneuronally innervated junctions after activity block than partially occupied junctions at the time the block was imposed, SN axons must have eliminated some LPN inputs. In other words, polyneuronally innervated endplates were not created simply as the result of regenerating SN axons filling vacant areas at partially innervated endplates, but by SN axons successfully competing to innervate regions of the endplates. Related to this is the difference between the fraction of the endplates that was unoccupied (at partially innervated endplates) 14 days after SN crush, and the fractional occupancy of the SN (at the polyinnervated endplates) after activity block. At the time the activity blocks were

imposed, the largest vacant area observed at the partially occupied endplates was 20% (see **Fig 3.12**). However, at the polyneuronally innervated endplates, the proportion of SN innervation was distributed across the range of fractional occupancies (from 12.7-97.2% of total endplate area in the TTX blocked muscles, and 4.5-94.8% of endplate area in the TTX/ α -BTX blocked group; see **Figs 3.17 & 3.21**) in all the muscles studied.

Thus, since 99.9% of endplates were 80% or more occupied by the LPN at the time regenerating SN axons returned to 4DL muscles, it is concluded that the vast majority of the observed SN innervation following paralysis was the result of activity-independent synapse elimination.

3.9.1 Axonal Damage During Dissection

It is possible that the failure of 4DL muscles to respond to SN stimulation *in vitro* 14 days after SN crush was not because regenerating SN axons hadn't returned to the muscle, but because SN axons were damaged during the nerve dissection. However, this possibility can be ruled out as extremely unlikely, given that muscles in the TTX group and their contralateral controls did contract in response to SN stimulation *in vitro* (28 days after SN crush). The observed stimulation-induced contraction provided a control for the dissection technique because there is no reason to suspect damage would have been done routinely in the 14 day SN crush experiments, but not in the activity block and contralateral control experiments. That the muscles contracted to nerve stimulation 28 days after SN crush provided further support that SN axons had not reached 4DL muscles 14 days after crush.

3.10 Failure to Determine the Presence of LPN Inputs, In All Cases

It is also possible that junctions which appeared to be innervated exclusively by axons belonging to the SN were, in fact, polyneuronally innervated, but LPN terminals failed to be stained with RH414. Any failure to stain may have reflected the presence of inactive inputs at the junctions, or nerve block, caused by hypoxia during dissection, or some pharmacological effect of the RH414 (Bewick & Betz, 1994). This point is not trivial: there is some evidence that the last indication a nerve terminal is being eliminated from an endplate is its failure to release neurotransmitter effectively (Colman et al., 1997). If such a mechanism was operating in some LPN axons, it would have been important to distinguish it from simple nerve conduction block. However, immunocytochemical staining didn't reveal the presence of any boutons at SN mononeuronally innervated junctions that hadn't previously been stained with FM1-43 or RH414 (see **Fig 3.19**). Although immuno-staining was not used to verify the innervation patterns of all the junctions with SN input in the series of experiments, immunocytochemistry nonetheless helps to provide confidence the inputs to the muscle fibres examined were active.

3.11 Efficacy of the Activity Block

3.11.1 TTX

It would be possible to explain the presence of SN mononeuronally innervated endplates if the TTX superfusion was ineffective or there were periods when TTX did not reach the sciatic nerve. Differences in SN and LPN activity at these times could then account for the observed synapse elimination (Balice-Gordon &

Lichtman, 1993, 1994). However, there are two indications that this was not the case. First, it was established that the nerve conduction block was effective from the time of the TTX implant to the time the muscles were examined experimentally. All animals were carefully monitored every day for clinical signs of paralysis in the treated limb and were only included in the analysis if they had been continuously paralysed for fourteen days. Second, upon sacrifice, there was no evidence of propagated activity when the sciatic nerve was cut above the cuff. If the TTX differentially affected axonal supplies, there should have been contractions in some of the motor units of the treated limb. In contrast, cutting the sciatic nerve below the cuff elicited vigorous contractions in all the musculature of the paralysed limb. The simplest explanation for this observation was that centrally conducted action potentials had been propagated up to the position of the cuff. Therefore, the daily behavioural tests for the presence of paralysis probably provided a reliable indication of effective nerve conduction block. From this discussion, it appears that the TTX abolished evoked transmitter release in both the SN and the LPN and the findings are thus not attributable to unequal or irregular sciatic nerve conduction block.

However, as discussed in the Introduction to this chapter, TTX does not abolish spontaneous activity and the possibility that MEPPs contributed to the observed synapse elimination in the TTX blocked muscles cannot be ruled out entirely. Indeed, the analysis of LPN/SN fractional occupancies at the polyneuronally innervated junctions suggests MEPPs did contribute to synapse elimination: SN inputs were significantly larger than convergent LPN inputs in the TTX blocked

muscles and controls, but there was no significant difference between the sizes of convergent inputs in the TTX/ α -BTX blocked muscles (see **Figs 3.20 & 3.22**). The simplest explanation for this difference is that spontaneous activity provided a competitive advantage for the regenerating SN inputs, at least under conditions of normal activity and TTX activity block. It has long been known that following denervation, muscle fibres become supersensitive to Ach (Brown, 1937): a denervated skeletal muscle is approximately 1000 times more sensitive to Ach applied either directly in the bathing fluid, or injected into an artery supplying the muscle than is a normally innervated muscle. The increased sensitivity results in superthreshold responses to levels of Ach that would otherwise have been subthreshold for activation of the muscle fibre. Denervation supersensitivity can also be produced without interrupting the nerve; blockage of synaptic activation of the muscle with TTX or α -BTX is sufficient (Berg & Hall, 1975; Witzemann et al., 1991) and the onset of supersensitivity is normally within 2-5 days of denervation or paralysis (Lømo & Rosenthal, 1972). In the present (TTX block) experiments, prolonged paralysis may have rendered the muscle fibres supersensitive to Ach, and it is therefore possible that differences in the timing of MEPPs from SN/LPN inputs constituted a sufficient level of activity to trigger synapse elimination. Consistent with this idea, Gundersen (1990) showed that MEPPs triggered muscle action potentials at 30% of extensor digitorum longus fibres during TTX paralysis. MEPPs from control muscles, which were not blocked with TTX, did not trigger action potentials.

However, any activity occurring in muscle fibres (other than evoked activity) has previously been ruled out as unlikely to be responsible for synapse elimination during paralysis. Sometimes, after denervation, individual muscle fibres start to exhibit spontaneous, asynchronous contractions called fibrillation. Fibrillation is caused by changes in the muscle membrane itself and is not initiated by Ach (Purves & Sakmann, 1974; Fambrough, 1979), although some of the spontaneous action potentials that produce fibrillation do originate in the region of the end plate (Belmar & Eyzaguirre, 1966). The level of endogenous activity in the fibres caused by fibrillation is not sufficient to overcome denervation-associated changes in membrane properties, for example, Ach supersensitivity (Purves & Sakmann, 1974). Ribchester (1993) suggested that since fibrillation is not sufficient to reverse the effects of denervation, any spontaneously generated muscle fibre action potentials caused by MEPPs becoming suprathreshold are probably also insufficient. Thus, the level of activity in muscle fibres by any means other than evoked release probably fails to account for synapse elimination during TTX blockade. Consistent with this idea, there were no significant differences between the overall amount of SN innervation in the three conditions in the present experiments (see **Fig 3.16**); it is difficult to imagine how spontaneous activity could give rise to greater SN fractional occupancy at polyneuronally innervated junctions without also bringing about more SN mononeuronally innervated junctions. In addition, there were no significant difference between the sizes of the SN inputs at polyneuronally innervated endplates in the TTX muscles compared to TTX/ α -BTX muscles or contralateral controls (see **Fig 3.20**). Thus, the reason for the increased size of SN inputs at polyneuronally innervated junctions in the TTX blocked muscles is unclear. There is variability in

the numbers of motor units supplied to the 4DL by the SN (Betz et al., 1979) and it is possible that the muscles included in the TTX block group had more SN motor units than those in the TTX/ α -BTX group. This may have given SN axons a competitive advantage in terms of the numbers of former synaptic sites the axons could reoccupy, without being effective enough to transform more of the SN-dominant polyneuronally innervated junctions into SN mononeuronally innervated endplates. A comparison of the numbers of motor units in TTX vs. TTX/ α -BTX blocked muscles was not possible because of the receptor blockade in the latter group, and so this explanation remains speculative.

There remains the possibility that TTX exerted slight toxic effects on the LPN, placing its terminal boutons at a competitive disadvantage. However, the presence of SN-only and polyneuronally innervated junctions in the contralateral control muscles suggests that any LPN competitive disadvantage was not caused by the TTX.

3.11.2 Unlabelled α -BTX Injections

The discussion above argued against the possibility that spontaneous activity accounted for the observed synapse elimination in the TTX blocked muscles. The finding that synapse elimination also occurred in the TTX/ α -BTX group strengthens this contention. Of course, this rests on the conditions that the daily unlabelled α -BTX injections (1) reached the 4DLs, (2) bound to all the receptor plaques in the muscles, and (3) blocked activity completely between injections. The results of control experiments described here imply that all of these conditions were satisfied.

With respect to the first condition, there have been several reports that injecting unlabelled α -BTX produces neuromuscular blockade of skeletal muscle. In the earliest studies, α -BTX was injected into the thoracic cavities of rats, where muscle fibres in the diaphragm were successfully blocked (Berg & Hall, 1975a and b; Chang et al., 1975). The results of the control experiments described here, using TRITC- α -BTX (see **Figs 3.3, 3.5, 3.6 & 3.8**) and electrophysiological measures (see **Figs 3.4 & 3.7**), agree with these earlier findings, suggesting that α -BTX successfully diffused to the 4DL from the site of injection. After trying several protocols for injections, Berg & Hall (1975b) noted that the optimum dose of α -BTX for blocking receptors in rats, while still offering the animals reasonable chances of survival, was in the order of 7 μ g/100g of body weight, every 12 hours. They found that no more than 70 μ g should be administered to any one animal in the course of an experiment (Berg & Hall, 1975a). This successful protocol was similar to the one used in the present experiments, in which rats were given 5 μ g of unlabelled α -BTX every 24 hours for 2 weeks (for a total of 70 μ g per animal).

Concerning the second condition, it is unlikely that the unlabelled α -BTX bound to some endplates in the muscle, while sparing other endplates. When TRITC- α -BTX was bathed (see **Fig 3.5**) or injected over (see **Fig 3.6**) 4DL muscles which had previously been given unlabelled α -BTX injections, no fully stained receptor plaques were detected. The lack of TRITC staining suggests that the receptor plaques in the muscles were already occupied by unlabelled α -BTX.

Concerning the third condition, there was some evidence that new AchRs were inserted into muscle fibre membranes between injections (see **Fig 3.8**). The half life of junctional AchRs that have already bound α -BTX is 7.5 days (Chang & Huang, 1975). However, the half life of Ach receptors which are inserted into denervated or paralysed muscle is just 24 hours (Shyng & Salpeter, 1990). Although it is possible that spontaneous release could have acted on newly synthesised receptors and triggered synapse elimination between injections, it is probably unlikely. First, as has been discussed above, the contribution of spontaneous activity to synapse elimination in the TTX blocked muscles was probably minimal. Second, the pattern of punctate staining at the junctions (see **Fig 3.8**) showed that only small regions of the receptor plaque failed to be blocked by unlabelled α -BTX. It would have to be argued that, not only did MEPPs trigger muscle action potentials at these endplates, but they triggered action potentials by activating less than 50% of the receptor plaque. It has been shown that EPPs of reduced amplitude can be elicited when 40% of an endplate's receptors have been blocked with subcutaneous α -BTX injections (Plomp et al, 1992). Another study showed that EPPs could be elicited from junctions when up to 80% of the receptor plaque was blocked by α -BTX (Chang et al., 1975). However, these estimates are indirect, and the fraction of the endplate that can be reliably blocked by α -BTX without abolishing the synaptic response still remains to be directly established. In the present experiments, the proportion of the muscle which was positive for punctate TRITC- α -BTX staining after unlabelled injections (see **Fig 3.8**) was only 1.4%. Assuming that this represented the fraction of muscle fibres which were subject to MEPP-dependent synapse elimination, the

figure is still too low to account for the level of SN innervation (16%; see **Fig 3.16**) seen at the end of paralysis.

To summarise, the analysis of these control experiments indicates that injections of α -BTX successfully blocked AchRs for the duration of the TTX activity blockade.

3.12 Activity-Dependent SN Reinnervation

A further explanation for the results could be that SN axons regenerated and recovered some level of innervation before the activity block was administered. However, regenerating axons grow at the rate of approximately 3mm/day (Jacobsen & Guth, 1965) and the distance between the site of the SN crush and the 4DL is approximately 50mm. Thus, it takes about 16 days for regenerating SN axons to return to the 4DL muscle. In addition, Ribchester (1993) manipulated the interval between SN crush and activity block. He found that when activity block was initiated as early as 10 days after SN crush, subsequent SN only innervated endplates were still detected in 4DL muscles. Consistent with this report, in the experiments described here, there was no muscle response to SN stimulation *in vitro* 14 days after SN crush. This provided a rough indication that regenerating axons had not reinnervated any 4DL fibres at the time the sciatic nerve was blocked.

3.13 Summary

Taken together, the present results suggest that crushing the SN leads to degeneration and withdrawal of injured nerve terminals while intact, uninjured LPN terminals rapidly attempt to re-innervate nearby denervated fibres. Compensatory

sprouting and innervation by the LPN is, on the whole, complete before regenerating SN axons return to 4DL muscles. The efficacy of the TTX block and the patency of the α -BTX block have been shown to be sufficient providers of muscle paralysis, and the contribution of spontaneous activity to synapse elimination is, at best, only limited. Thus, the most plausible way to account for the presence of SN innervation after activity block is if SN axons eliminated intact and/or sprouted LPN inputs by activity-independent mechanisms.

3.14 Activity-Independent Synapse Elimination

These results agree with a small number of studies that have reported synapse elimination at inactive neuromuscular junctions. When Thompson et al. (1979) applied TTX to convergent inputs during neonatal synapse elimination, they noted that during the first 2-4 days of activity block, polyneuronal innervation declined in the muscles as normal. Brown et al. (1981) confirmed this finding in several neonatal skeletal muscles, by blocking all activity with botulinum toxin injections. In both cases, longer periods of paralysis produced increased levels of polyneuronal innervation which were not transformed to mononeuronal patterns by the end of the developmental period of synapse elimination. However, because activity was blocked after synapse elimination had already begun in both of these studies, it was unclear whether the initial loss of polyneuronal innervation reflected inputs which were already in the process of being eliminated prior to the activity block. Using the electrophysiological and histological techniques available to them at the time, the authors had no way to determine whether synapse elimination was abolished completely during activity blockade, or continued during activity block, but was

exceeded by the rate of synapse formation. The present experiments help to clarify this issue: TTX activity block was imposed *before* regenerating axons reinnervated the muscles, so there was no confounding effect of earlier activity-dependent mechanisms perpetuating synapse elimination. The results suggest that synapse elimination does take place in paralysed muscle, but is masked by excess synapse formation. Thus, it appears that synapse formation and elimination may be concurrent on-going processes in paralysed muscle, as has been described previously in partially denervated muscle (Ridge & Rowlerson, 1990).

Ribchester (1988) and Barry & Ribchester (1995) have also presented evidence that regenerating axons eliminated intact axons in the absence of activity. Only 3 motor units are required to fully innervate the 4DL following partial denervation (Ribchester, 1988). In both studies, 4DL muscles were subjected to LPN crush and TTX activity block; the authors found in both cases, that in all muscles where the SN provided 3 or more motor units, there were fibres occupied solely by axons from the LPN. However, like the Thompson et al. (1979) and Brown et al. (1981) studies, in the Ribchester (1988) and Barry & Ribchester (1995) experiments, reinnervation and activity were allowed to progress in the muscle prior to the imposition of the TTX activity block.

The results most closely agree with Ribchester's (1993), which described the ability of the (minority) SN to eliminate intact LPN inputs during TTX activity block. Consistent with the results described in this chapter, there were endplates that were mononeuronally innervated by the SN in all the muscles included in Ribchester's

study. The major weakness of that study was that it failed to show conclusively that the 4DL was fully occupied by the LPN before the SN returned to the muscles. The results of the experiments with immunocytochemical staining (section 3.6) provide this evidence. In addition, the experiments with α -BTX injections show unequivocally that synapse elimination can take place independently of activity.

The results disagree sharply with two studies. In the first soleus muscles were treated with α -BTX during neonatal synapse elimination (Duxson, 1982). In contrast to all of the reports discussed above, postsynaptic blockade rapidly suspended the retraction of supernumerary axons, essentially fixing the synapses into place. The evidence for this was that there was no significant difference between the numbers of small, medium, and large terminal boutons at the time of, and 2 days after neuromuscular block. Duxson argued that concurrent terminal sprouting and retraction could not explain the results, because a shift in the numbers of boutons would be expected, with an increase in the number of the smallest boutons (due to sprouting) and a fall in the number of large and medium boutons (as excess axons retracted). However, a closer inspection of the data reveals that there was an increase in the overall numbers of boutons/endplate after activity block. Duxson reported the difference to be non-significant at the 1% level, but when the data are tested at the 5% level, the difference *is* significant. The increase in the numbers of boutons after activity block can only be explained by sprouting and so it is still possible to explain the data by the elimination of small boutons, and the addition of sprouted axon terminals.

The present results also seem to disagree with a finding by Balice-Gordon & Lichtman (1994), who concluded that asymmetry in the pattern of muscle fibre activation by convergent inputs triggers synapse elimination. They reported that

“block of 100% of the AchRs in 30% (or less) of the junction induces [synapse elimination], whereas blocking 30% of the AchRs in 100% of the junction is ineffective”.

The major weakness of this observation is that the authors did not determine whether blocking 30% of AchRs with α -BTX actually blocked synaptic transmission. Chang et al (1975) showed that EPPs could be elicited from junctions when up to almost 80% of AchRs were blocked with α -BTX. Although indirectly obtained, Chang et al's results cast some doubt on the extent to which Balice-Gordon & Lichtman's conclusions can be safely made, on the basis of their experimental paradigm.

3.15 Differential Activity

The foregoing discussion focused on how the outcome of synapse elimination is affected when all the inputs to a muscle fibre are paralysed. Another experimental paradigm that has been used extensively involves pitting active (or more active) and inactive (or less active) motor units against one another to ask if inputs are advantaged either way during synapse elimination. Although the paradigms differ from the one under investigation here, the results deserve brief comment, because some suggest that inactive synapses eliminated active synapses.

There is, as yet, no consensus as to the outcome of synapse elimination under conditions of differential activity. For example, Ribchester & Taxt (1983, 1984)

showed that active motor units were favoured over inactive units during reinnervation of 4DL muscles. Likewise, selective stimulation of motor units in neonatal muscles gave a competitive advantage to the stimulated axons (Ridge & Betz, 1984). Intracellular recordings from isolated neuromuscular junctions (Betz et al., 1989) and cultured myocytes (Lo & Poo, 1991; Dan & Poo, 1992) revealed synaptic suppression of non-stimulated inputs that were in close proximity to inputs which had been stimulated. Finally, Balice-Gordon & Lichtman (1994) did show that nerve terminals overlying small regions of blocked AchRs were removed when the remainder of the receptors were active. All of these results indicate that synapses are strengthened when active, and weakened when inactive, suggesting a competitive advantage for the most active inputs.

However, directly contradictory results have also been obtained (Callaway et al., 1987, 1989). Blocking electrical activity with TTX in one of three ventral roots innervating rabbit soleus muscle resulted in larger motor units from the nerve with activity blockade than from converging axons from nerve roots in which electrical activity was not blocked. Nelson et al. (1993) selectively stimulated convergent inputs to polyneuronally innervated junctions in culture and found that both the stimulated and unstimulated inputs to the muscle cells exhibited synapse elimination to a statistically indistinguishable extent. In contrast to the studies described immediately above, these results all suggest a competitive advantage to inactive synapses.

3.16 Possible Mechanisms for Activity-Independent Synapse Elimination

There are several plausible ways in which inactive synapses might be benefited during synapse elimination. A relatively straightforward mechanism could be based on a tendency for activity to suppress terminal sprouting, much as has been reported for invertebrate neurons in tissue culture (Cohan & Kater, 1986), and for inactivity to enhance terminal sprouting (Brown et al., 1980). Accordingly, the results of Callaway et al. (1987, 1989) and Nelson et al. (1993) could be explained by the inactive terminals initiating more contacts with muscle fibres and establishing more sites for competition to take place, while active terminals received no incentive (signal) to sprout. Likewise, in this chapter's experiments, even though the LPN had sprouted fully before the SN regenerated, continued inactivity would *also* have stimulated the SN to sprout and thereby enter effectively into competition with LPN terminals.

A more indirect route might be based on a tendency for activity to down-regulate the release of a trophic factor by muscle fibres (reviewed by Purves, 1986). Inactive motor neurons could gain a small advantage in acquiring this factor because the population of muscle fibres they innervate has an activity level slightly lower than that of normally active motor neurons. In the experiments described here, the prolonged inactivity may have resulted in the prolonged production of one or more trophic factors so that the regenerating SN axons acquired access to it/them. One prediction of such a mechanism would be an increased overall amount of synapse formation and polyneuronal innervation in the muscles. The results described here are consistent with this prediction (see **Fig 3.16**).

A great deal of research is directed toward the search for products which function as specific trophic factors in skeletal muscle. *Neurotrophic theory* (Purves, 1988) posits that neurons are supported by substances which are limited in supply, and the number of neurons innervating a target is regulated according to supply and demand. The molecular understanding of the function of neurotrophic factors began with the discovery of nerve growth factor (NGF), and its effects on the survival and growth of neurons in culture (Levi-Montalcini et al., 1954). The search for other factors with similar actions on neurons has led to the discovery of a family of NGF-like molecules, each specific for different populations of neurons. Other members of this family now include brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4/5, and NT-6 (reviewed by Lewin & Barde, 1996).

The involvement of NGF-like molecules, and the related molecules ciliary neurotrophic factor (CNTF) and glial cell line-derived neurotrophic factor (GDNF) in neuromuscular plasticity have been suggested for several reasons. First, many of these factors can enhance the survival of embryonic motor neurons *in vitro* (Henderson et al., 1993, 1994; Zurn et al., 1996) and some prevent axotomy-induced or programmed cell death *in vivo* (BDNF: Yan et al., 1992; Oppenheim et al., 1992; CNTF: Oppenheim et al., 1991; NT4/5: Koliatsos et al., 1994). Second, the mRNAs encoding various neurotrophic factors are present in skeletal muscle during various stages of development (Maisonpierre et al., 1990; Henderson et al., 1993; Koliatsos et al., 1993), BDNF mRNA is upregulated after denervation (Funakoshi et al., 1993; Koliatsos et al., 1993), and the mRNA for neurotrophic receptors is present in motor neurons (Funakoshi, et al., 1993; Henderson et al., 1993; Koliatsos et al., 1993).

Finally, BDNF and NT-3 can potentiate transmission at developing neuromuscular synapses by increasing the frequency of spontaneous synaptic currents and by increasing the amplitude of evoked synaptic currents in tissue culture (Lohof et al., 1993). Recently, however, these effects of BDNF and NT-3 were not replicated at slightly older neuromuscular junctions *in vitro*, although GDNF did produce a potentiation of transmission (Ribchester et al., 1998).

The reason for the contradiction is unclear, but it may be that growing axons respond more effectively to lower doses of some neurotrophic factors (see Ribchester et al., 1998). Computer simulations and theoretical/mathematical analysis of the way neurotrophins influence the upregulation of their specific receptors on growing axons have demonstrated that the capacity of a growing axon to obtain access to a factor determines which inputs will innervate the motor endplate (Rasmussen & Willshaw, 1993; van Ooyen & Willshaw, 1999). This type of mechanism may have provided a competitive advantage for regenerating SN axons in the present experiments. These studies raise the possibility that any competitive advantage of regenerating SN axons may need to be considered within the context of several coincident signals. For example, a recent report (Boulanger & Poo, 1999) showed that a brief depolarisation in the presence of BDNF resulted in the potentiation of evoked and spontaneous synaptic transmission, whereas exposure to BDNF alone, or depolarisation alone, had no effect. Quantal depolarisations occur when growing axons come into contact with muscle fibres (Chow et al., 1988; Tabti & Poo, 1990). The spontaneous release from regenerating SN axons was unaffected in the present experiments; it may be that these brief depolarisations in

conjunction with the presence of one or more neurotrophic factors gave SN axons a competitive, presynaptic, advantage in reinnervating the 4DL muscles. This type of effect has been described in developing tfl muscles: BDNF, NT-3 and NT-4/5 all caused the retention of polyneuronal innervation, but BDNF was further shown stabilise both functional and electrically silent synapses (Kwon & Gurney, 1996). Interestingly, in these experiments, NGF had no effect on synapse elimination, suggesting that different neurotrophic factors may influence different aspects of neuromuscular development and plasticity.

3.17 Future Work

The results presented here raise several possibilities for future avenues of investigation. First, it seems that the results are in contradiction to Hebbian (1949) rules of synaptic plasticity, which essentially state that synapses in which pre-and postsynaptic activity are correlated will be strengthened (and maintained) and synapses in which the activity is not correlated will be weakened (and lost). Here, even though the pattern of (in)activity was correlated at all the endplates, synapses were still eliminated. Other studies of synchronised, correlated activity have also shown that synapse elimination takes place in contravention of Hebbian rules (O'Brien et al., 1978; Thompson, 1983). Indeed, in these studies, the time course of synapse elimination was even reported to have been accelerated. However, it is not known whether the synchronous activity imposed in these experiments was in addition to naturally occurring asynchronous patterns of activity. The present experimental technique could help to clarify this question: by imposing synchronous activity on 4DL muscles concurrent with TTX block, pre-and postsynaptic activity

could be directly controlled. Any synapse elimination that occurred as the consequence of such a paradigm would further demonstrate an influential, rather than a decisive role for activity in neuromuscular plasticity.

Second, part of the discussion above focused on diffusible factors which may be released by the muscle in response to denervation and/or paralysis, and which have effects on synaptic structure and function. If, as has been suggested previously, growing/regenerating axons have a competitive advantage over intact axons because of preferential access to a neurotrophic factor, a study at the electron microscopic (EM) level could help to confirm or refute this possibility. SN and LPN axons could be labelled for receptors to a given neurotrophic factor and any increase in the density of the receptor found on SN axons may suggest an advantageous role for that factor in synapse elimination.

Finally, an investigation into the role of electrical fields in stimulating and guiding synaptic rearrangements may be attempted using the current paradigm. Based on the finding that perimembrane electric fields may move and induce aggregation of AchRs (Orida & Poo, 1978; Poo, 1981), electrokinetic redistribution of receptors in the plasma membrane by synaptic currents has been proposed as a potential mechanism for strengthening active synapses and weakening inactive ones (Poo & Young, 1996). The highest level of activity block achieved in the present experiments was to inhibit spontaneous and evoked activation of the AchRs. However, spontaneous release was not affected, and it is possible that the release of Ach influenced the strength of synapses, as described in these earlier studies. To

determine if there is any competitive advantage lent to axons by electrochemical gradients, the present paradigm could be adapted to also block all release of Ach; Botulinum Toxin, by preventing the docking of vesicles in the nerve terminal is known to produce such an effect (Montecucco & Shiavo, 1993).

Chapter 4:
Structure:Function Correlates of Persistent
Polyneuronal Innervation

4.1 Introduction

The mechanisms controlling the elimination of polyneuronal innervation in developing or reinnervated skeletal muscle is unknown. A body of experimental evidence has suggested that there are stereotyped cascades of structural and functional changes associated with synapse elimination, the rate and outcome of which are controlled by activity (Balice-Gordon & Lichtman, 1994; Colman et al., 1997). The interpretation of these results rests on the premise that polyneuronal innervation is a highly unstable pattern of innervation, and synapse elimination will be triggered by differences in the timing of AchR activation by convergent inputs to muscle fibres, whenever endplates are not mononeuronally innervated (Frank, 1997). However, other studies show that polyneuronal innervated endplates can persist, perhaps indefinitely, in skeletal muscle (Barry & Ribchester, 1995), suggesting that activity may *not* be sufficient to trigger synapse elimination at all neuromuscular junctions.

One possible way to reconcile these differences is to hypothesise that the “persistent” polyneuronal inputs observed by Barry & Ribchester (1995) were, in fact, engaged in synapse elimination, but on a slower time course than expected. If this was the case, there should have been evidence that the small inputs at convergently innervated endplates were being removed. Other studies predict that there should have been a visible reduction in the density of AchR clustering (Rich & Lichtman, 1989; Balice-Gordon & Lichtman, 1993,1994) and/or a reduction in transmission efficacy (both quantal size and quantal content; Colman et al., 1997), associated with inputs in the process of synapse elimination. These types of correlational data suggest that

smaller inputs are eliminated because they are disproportionately weaker than large inputs: small inputs produce a smaller synaptic effect because the amount of neurotransmitter released over the corresponding area of receptors is absolutely less than the amount released by a large input. However, the effect is also disproportionate weakness because an input in the process of being eliminated releases progressively less transmitter over a progressively reduced area of AchRs (Frank, 1997). One prediction of this explanation of synapse elimination is that if the synaptic strengths of small and large inputs were compared on a per unit area basis (for example, quantal content per square micron) a small input would be weaker than a large input. However, the relationship between convergent inputs has never been directly measured in mammalian skeletal muscle, and so it is unknown if small inputs are weaker than large inputs and if so, in what capacity: are they proportionately or *disproportionately* weaker.

The experiments described in this chapter were designed to (1) clarify the relationship between “persistent”, convergent inputs at mammalian skeletal endplates, and (2) detect evidence that the small inputs are in the process of being eliminated. Two hypotheses were tested. First, small inputs are synaptically weaker than large inputs at polyneuronally innervated endplates. Second, small inputs are also disproportionately weaker than their contiguous neighbours. Synaptic strength was assessed in terms of overall effect (mV of depolarisation of the muscle fibre) and the amount of transmitter released (quantal content).

4.2 Experimental Design

The paradigm used to produce persistent polyneuronal innervation was identical to that used by Barry & Ribchester (1995; Fig 4.1). Fourth deep lumbrical muscles were partially denervated by crushing the LPN bilaterally; 19-21 days later, activity was abolished in the right sciatic nerve with a TTX implant. Nerve block was maintained continuously for 14-34 days, and activity resumed in the muscles for 2-10 weeks (median, 6 weeks) before sacrifice. Nerve-muscle preparations and intracellular recordings of identified neuromuscular junctions were made as described previously (see sections 2.1, 2.4 & 2.5).

In the first series of experiments, animals were sacrificed 3 days after LPN crush, to ensure that degeneration of the injured terminals and sprouting of intact (SN) terminals took place. Nerve-muscle preparations were fixed and stained by immunocytochemistry to determine the effect of the partial denervation.

A note on terminology

Activity block was always imposed on the right hind limb, so only the right 4DL muscles were paralysed. These reinnervated, paralysed muscles will be referred to as the “experimental” or “TTX blocked” group of muscles. Contralateral control 4DLs were reinnervated only, as the consequence of bilateral LPN crush. These muscles will be referred to as the “control” group of muscles.

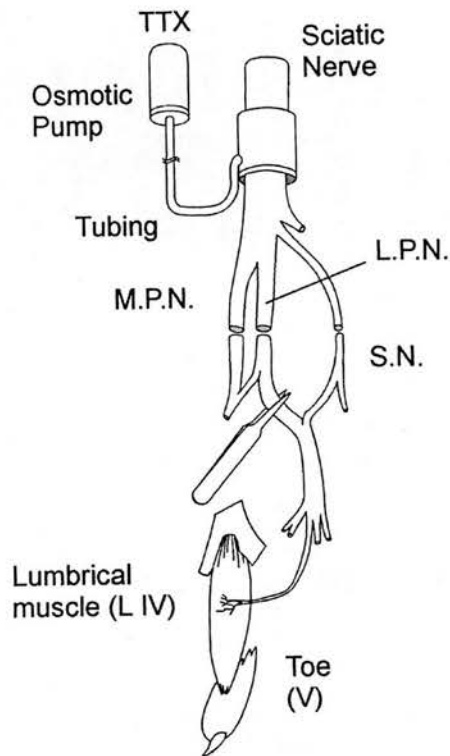


Fig 4.1 Experimental design. The forceps indicate the location of the LPN crush. Following 19-21 days recovery from nerve crush, an osmotic minipump was implanted in the peritoneum, and TTX was continuously superfused to the sciatic nerve for times ranging between 2-10 weeks. Animals were allowed to recover from the TTX activity block for a minimum of 2 weeks before sacrifice. *Reproduced, with permission, from Barry & Ribchester (1995).*

4.3 Measurements of Synaptic Strength

4.3.1 Effects of Dye Loading on Synaptic Transmission

High concentrations of RH414 can depress synaptic efficacy (Bewick & Betz, 1994). Because it was necessary to label nerve terminals before electrophysiological recording in the present experiments, it was verified that the concentration of RH414 used here did not affect LPN transmission. An experimental 4DL nerve-muscle preparation was made and a polyneuronally innervated endplate was found using intracellular recording. Baseline amplitudes of EPP responses were recorded from the LPN and SN. Nerve terminals were labelled with FM1-43 and RH414 in turn, as described in Chapter 2 (section 2.1.6) while the EPP amplitudes were monitored. Finally, EPP amplitudes were recorded for a further 10 minutes following dye washout.

4.3.2 Relationship Between Absolute Synaptic Size and Effect

In order to determine if small synaptic inputs were weaker than large inputs, the total synaptic effects of LPN and SN inputs at polyneuronally innervated junctions were compared. Here, effect refers to the mean EPP amplitude response (in mV) to 100 stimulations of the LPN or SN, corrected for non-linear summation to -80mV.

4.3.3 Relationship Between Relative Synaptic Size and Effect

To determine if small inputs were disproportionately weaker than large inputs, the sizes of convergent inputs were normalised, using the equation:

$$e:o = (\text{Effect}_i / \text{Effect}_T) * (\text{Area}_T / \text{Area}_i)$$

where i and T denote the input, and the summed total of the two inputs, respectively. The resulting, unitless value is referred to as the efficacy:occupancy (e:o) index.

4.3.4 Rise Times of Convergent Inputs

The MEPP amplitude and the time-to-peak of EPP amplitudes can be used as an indication of AchR density (Land et al., 1980; Kidokoro, 1980). Because it was unknown which input produced MEPPs at the polyneuronally innervated endplates, the time-to-peak of the evoked potentials was used as one indication of receptor function beneath the small inputs.

4.4 Measurements of Presynaptic Strength

4.4.1 Relationship Between Absolute Synaptic Size and Quantal Content

To determine if small synaptic inputs released less transmitter than large inputs, the quantal contents of LPN and SN inputs at polyneuronally innervated junctions were compared (see section 2.4).

4.4.2 Relationship Between Relative Synaptic Size and Quantal Content

To determine if small inputs released disproportionately less transmitter than large inputs, the sizes of convergent inputs were normalised, using the equation:

$$qc:o = (QC_i / QC_T) * (Area_T / Area_i)$$

where i and T denote the input and the summed total of the two inputs, respectively. The resulting, unitless value is referred to as the quantal content:occupancy (qc:o) index.

4.5 Measurements of Postsynaptic Strength

4.5.1 Physiological Analysis of AchR Density

High frequency nerve stimulation causes an increase in the frequency of MEPPs for a short period after the tetanisation ends (Colman et al., 1997). This property of nerve transmission was exploited to evaluate the function of AchRs beneath small vs. large inputs. Polyneuronally innervated junctions with divergent LPN and SN EPP amplitudes were located using intracellular recording. The nerves were tetanically stimulated in turn (10Hz for 30 seconds) and spontaneous activity was recorded for one minute following each tetanisation. The morphology of inputs was not identified in these experiments.

4.5.2 Morphological Analysis of AchR Density

In vivo synaptic imaging studies have demonstrated that TRITC- α -BTX is sensitive enough to reveal regions of the endplate where the density of AchR clustering is reduced (Rich & Lichtman, 1989; Balice-Gordon & Lichtman, 1993, 1994). In one series of experiments, AchR plaques at polyneuronally innervated junctions were labelled with TRITC- α -BTX to determine if the intensity of staining was diminished beneath small inputs.

In the first experiments, TTX-blocked muscles were stained with TRITC- α -BTX and receptor plaques were imaged, and assessed for the fluorescence intensity throughout the junctions. Nerve terminals were stained with FM1-43/RH414 and polyneuronally innervated endplates were relocated. Because this method produced a low yield of data, the paradigm was adapted. It was assumed that if a receptor

plaque contained a region of reduced density, the overall fluorescence intensity would be lower than in a plaque without a region of reduced density. Thus, in the second experiments, polyneuronal innervation was determined by immunocytochemical staining. AchRs were stained with TRITC- α -BTX and the fluorescence intensity throughout the receptor plaques was measured using OpenLab software. Background fluorescence was minimised by using a short integration time during image capturing, and using the same capturing settings between muscles.

Results

4.6 Effects of LPN Denervation on SN Sprouting

Three days after nerve crush, there was no muscle contraction in response to cutting the LPN, in any of the 8 nerve-muscle preparations studied *in vitro*. In 2 muscles, there was also no response to cutting the SN; these preparations were discarded from further analysis and are not included in the description of the results that follow.

In all of the remaining muscles in the group (n=6), the majority of the endplates were unoccupied ($68 \pm 4.4 \%$), indicated by AchR staining with no overlying SV2 staining (Fig 4.2).

Partially occupied endplates comprised the second most common pattern of innervation in the muscles. Immunocytochemical analysis revealed that $26 \pm 5\%$ of the endplates were partially occupied. The partially occupied endplates were characterised by regions of the receptor clusters that were not overlaid by nf and/or SV2 staining. There was variation in the fraction of the receptor patches that was unoccupied. Of 261 partially occupied endplates, the smallest fraction of the receptor plaque that was not covered by nerve terminal staining was $5.4 \mu\text{m}^2$ (representing 3% of the total area of receptor staining; Fig 4.3a) and the largest area was $124.5 \mu\text{m}^2$ (98.3% of total endplate area; Fig 4.3b). The mean proportion of the receptor plaques that was unoccupied was $38.2 \pm 3.6\%$.

SN terminal sprouting was apparent in all the muscles: $14.8 \pm 3.7\%$ of endplates received or projected a nerve sprout. Most of the sprouts ($10.2 \pm 2.7\%$) were projected from fully or partially innervated endplates towards nearby denervated endplates (Fig 4.4a). The remainder of the sprouts ($4.7 \pm 1.3\%$) had contacted neighbouring denervated fibres (Fig 4.4b).

Finally, the minority of the muscle fibres were fully occupied ($6.5 \pm 1.0\%$). Fully occupied endplates were indicated by a 100% overlap between the area of nerve terminal/axonal staining and the area of the receptor patch (Fig 4.5).

4.7 Morphology of Reinnervated, Paralysed 4DL Neuromuscular Junctions

There were similar patterns of innervation in all 16 experimental muscles studied: endplates that were polyneuronally innervated ($32.5 \pm 3.3\%$), and mononeuronally innervated by the SN ($24.2 \pm 1.8\%$), or LPN ($43.3 \pm 3.3\%$) were detected. In 17 control muscles, there were also SN and LPN mononeuronally innervated endplates ($29.5 \pm 1.9\%$ and $51 \pm 2.2\%$, respectively), as well as polyneuronally innervated endplates ($19.2 \pm 1.7\%$). The levels of polyneuronal innervation were significantly greater in the TTX blocked muscles than in controls ($P < 0.01$). The proportions of LPN and SN mononeuronally innervated endplates in the experimental muscles were not quite significantly different from controls ($P = 0.06$ for LPN and $P = 0.05$ for SN comparisons). In both groups, there was a range of fractional occupancies by the two nerves at the polyneuronally innervated junctions. Sometimes the SN was the dominant input (Fig

4.6a), sometimes the LPN was the major nerve supply (Fig 4.6b) and sometimes the two nerves supplied roughly equivalent areas of the endplate (Fig 4.6c).

In the experimental muscles (n=17), there were no significant differences between the endplate sizes of SN ($208.2 \pm 10.9 \mu\text{m}^2$), LPN ($209.4 \pm 13 \mu\text{m}^2$) or polyneuronally innervated endplates ($217.1 \pm 12.5 \mu\text{m}^2$; $P > 0.05$, one way ANOVA; Fig 4.7). The mean number of boutons per endplate were as follows: polyneuronally innervated: $26 (\pm 2.6)$; SN mononeuronally innervated: $18 (\pm 1.8)$; LPN mononeuronally innervated: $22 (\pm 2)$. There was no significant difference between the mean numbers of boutons per endplate between the three innervation patterns ($P > 0.05$; one way ANOVA; Fig 4.7). There were also no significant correlations between endplate size and number of boutons/endplate at the SN mononeuronally innervated ($r = 0.1$) or the polyneuronally innervated endplates ($r = -0.2$). However, there was a significant correlation between the number of boutons per endplate at the LPN mononeuronally innervated endplates ($r = 0.6$).

4.8 Synaptic Function Of Convergent Inputs At Polyneuronally Innervated 4DL Neuromuscular Junctions

4.8.1 Effects of Styryl Dyes on LPN and SN Transmission

There was no significant change from baseline in the LPN EPP amplitude after labelling boutons with RH414 (amplitude before tetanisation: $10.4 \pm 0.40 \text{ mV}$; amplitude after tetanisation: $10.3 \pm 0.23 \text{ mV}$; $P > 0.05$, paired t test; Fig 4.8a). Likewise, there was no change in the SN EPP amplitude before and after FM1-43 labelling (amplitude before

tetani: 4.3 ± 0.17 mV; amplitude after tetanisation: 4.6 ± 0.10 mV; $P > 0.05$, paired t test; Fig 4.8b).

4.8.2 Relationship Between Absolute Synaptic Size and Effect: Do Smaller Inputs at Polyneuronally Innervated Junctions Produce Smaller EPPs?

Eighteen polyneuronally innervated junctions were identified by FM1-43/RH414 labelling and then subject to focal intracellular recording under the fluorescence microscope. Of these junctions ($n=36$ inputs), three were innervated by unequal amounts of LPN and SN boutons (where one nerve contributed between 1-44% and the other contributed between 56-99% of total endplate area); 15 were innervated by roughly equivalent areas of LPN and SN boutons (where each nerve occupied between 45-55% of total endplate area). Intracellular recordings showed that polyneuronally innervated junctions supplied by approximately equal areas of LPN and SN terminals produced EPPs that were approximately equal in amplitude (Fig 4.9a). Junctions supplied by inputs differing substantially in size give larger responses to the larger of the two inputs and proportionately smaller responses to the smaller inputs, and this was found regardless of whether the LPN or the SN was the larger input (Fig 4.9b and c).

Small LPN and SN inputs were weaker than large inputs. First, there was a very strong correlation between the fractional occupancy and the fractional synaptic effect of the LPN inputs (Pearson's $r=0.96$; Fig 4.10a). For example, at one polyneuronally innervated junction, the dominant (LPN) input contributed 94% of total junctional size and 93% of total muscle fibre depolarisation. At another junction, the minor (LPN)

input contributed 6.8% of total junctional size and 6.2% of total muscle fibre depolarisation. The 95% confidence limits (0.86 to 1.16) indicated that the correlation was highly significant ($P < 0.0001$, Fig 4.10a). The mean difference between each LPN input's contribution to total junctional size and effect was 5.9% ($\pm 1.3\%$). The data was closely clustered around the regression line; the mean difference of the each LPN input's contribution to total synaptic effect and the regression line was 5.7% ($\pm 1.2\%$; *regression line slope*=1.0). Three of the 18 LPN inputs showed fractional contributions that were outside this range. These inputs contributed 11, 18 and 22% less to synaptic effect than expected, based on their sizes (for example, one input contributed 81% of total area but just 59% of total synaptic effect).

In all the experiments in which the effect of one nerve is measured as a fraction of the effects of both nerves jointly, the same data can be plotted in one of two ways: either in terms of the percentage effect of the LPN input or in terms of the percentage effect of the SN input. The two plots generated have different forms but are entirely equivalent as in all cases the percentage LPN effect together with the percentage SN effect sum to unity. In this case, figures 4.10c and 4.10d (for SN) show the same data shown in figures 4.10a and 4.10b (for the LPN).

4.8.3 Relationship Between Relative Synaptic Size and Effect: Do Smaller Inputs at Polyneuronally Innervated Junctions Produce Disproportionately Smaller EPPs?

Small LPN and SN inputs at polyneuronally innervated junctions were not disproportionately weak. First, when LPN inputs were normalised for size, there was no significant correlation between fractional occupancy and synaptic effect (Pearson's $r=0.03$; *confidence limits*: -0.23 to 0.65; Fig 4.10b). For example, at one polyneuronally innervated junction, most of the area was supplied by the LPN (94%); at another endplate, the LPN innervated just 21%. However, the synaptic effects of the two inputs were 0.97 and 1.1, respectively. There was very little scatter in the data, with most of the points clustered around the regression line. The mean difference between each LPN input's e:o index and the regression line was 0.17 (± 0.03 ; *regression line slope*=0.002).

Figure 4.10d shows the same data, expressed in terms of the relationship between SN e:o index and SN percentage occupancy (SN e:o index is the reciprocal of the LPN e:o index; see section 4.3.3).

4.8.4 Rise Times of Endplate Potentials

The mean rise time of EPPs from the small LPN inputs (<50% of total endplate size) was 1.92 (\pm 0.20) msec, and the mean rise time for the large LPN inputs was 1.6 (\pm 0.20) msec. There was a weak, negative correlation between the rise times and fractional occupancies of the LPN inputs (Pearson's $r = -0.28$; Fig 4.11), however, this was not significantly different from zero ($P > 0.05$). The mean rise time of EPPs belonging to the small SN inputs was 1.49 (\pm 0.31) msec and the mean rise time for the large SN inputs was 1.32 (\pm 0.18) msec. The difference between the two groups was not significant ($P > 0.05$; Fig 4.11). Overall, a range of rise times was recorded from both the largest and smallest LPN and SN inputs.

4.9 Presynaptic Function Of Convergent Inputs At Polyneuronally Innervated 4DL Neuromuscular Junctions

4.9.1 Relationship Between Absolute Synaptic Size and Quantal Content: Do Small Inputs Release Less Neurotransmitter Than Large Inputs?

There were strong correlations between the fractional occupancy of LPN/SN inputs and their contributions to the total amount of neurotransmitter released at the junctions in response to nerve stimulation. First, small LPN inputs released significantly less transmitter than large LPN inputs (Pearson's $r = 0.78$; Fig 4.12a). However, the correlation was not as strong as for the fractional occupancy and overall synaptic effects of LPN inputs (see above and Fig 4.10a). Nonetheless, the 95% confidence limits (0.50

to 0.91) indicated that the correlation was significant ($P < 0.0001$). There was more scatter in the data than for the data showing LPN fractional occupancy and overall synaptic effect. Here, the mean difference between each LPN's size and contribution to quantal content was 10.1% ($\pm 3.9\%$) and the mean difference between each input's contribution to quantal content and the regression line was 11.0% ($\pm 1.9\%$; *regression line slope*=0.65). However, only four inputs fell outside this range; all four of these inputs contributed more to total junctional neurotransmitter release than would be expected, based on their sizes. For example, at one polyneuronally innervated junction, the minor (LPN) input provided only 12% of total size junctional size but 66% of the total junctional quantal content.

Figure 4.12 shows the same data, expressed in terms of the relationship between SN quantal content and percentage SN occupancy.

4.9.2 Relationship Between Relative Synaptic Size and Quantal Content: Do Small Inputs Release Disproportionately Less Neurotransmitter Than Large Inputs?

When junctions were normalised for size, negative correlations between the amount of neurotransmitter released at small vs. large LPN and SN inputs were revealed. First, small LPN inputs released less neurotransmitter per square micron than large LPN inputs (Pearson's $r=-0.5$; Fig 4.12b). The 95% confidence limits (-0.79 to -0.06) indicated that this correlation was significantly different from zero ($P<0.05$). There was a considerable amount of scatter in the data, particularly for the smallest LPN inputs. The mean difference between each LPN input's qc:o index and the regression line was $0.65 (\pm 0.16; \text{regression line slope}=-0.02)$, with several of the smallest inputs showing qc:o indices which were disproportionately high. For example, the smallest LPN input in the data set supplied only 12.0% of total junctional size, but had an qc:o index of 5.4, the largest index in the data set.

Figure 4.12d shows the same data, expressed in terms of the relationship between SN qc:o index as a function of percentage SN occupancy.

4.10 Postsynaptic Function Of Convergent Inputs At Polyneuronally Innervated 4DL Neuromuscular Junctions : Acetylcholine Receptor Efficacy

4.10.1 Characteristics of Spontaneous Release Events (MEPPs)

There were no significant differences between the spontaneous activity of large and small inputs at polyneuronally innervated junctions in which the amounts of LPN and SN innervation were unequal. Table 4.1 and Fig 4.13 summarise the data showing that the frequency and amplitude of MEPPs were similar beneath both inputs, regardless of which nerve provided the larger EPP at a junction.

4.10.2 Density of Ach Receptor Clustering

There was no qualitative difference in the intensity of AchR staining under small synaptic inputs compared to larger neighbouring inputs (Fig 4.14; n=2 relocated junctions).

When innervation patterns were determined by immunocytochemical staining (Fig 4.15), the mean pixel intensity of the Ach receptors at the mononeuronally innervated junctions was 34.0 ± 0.68 (arbitrary units on a grey scale of 0 to 256; n=42) and the pixel intensity beneath the polyneuronally innervated junctions was 33.1 ± 0.83 (n=40); there was no significant difference between the two means ($P>0.05$).

Fig 4.2 The majority of 4DL endplates were vacant, 3 days after LPN crush. AchRs were stained with TRITC- α -BTX and nf/SV2 were immunolabelled with FITC antibodies, so regions of innervation and areas where axons cross the endplate appear yellow. The TRITC- α -BTX staining indicated the location of endplates on the muscle fibres. In these examples, FITC-positive labelling for nf demonstrated that the immunocytochemical staining was robust; there was no SV2-positive staining, indicating that there were no nerve terminal boutons present at these endplates. Scale bar is 20 μ m.

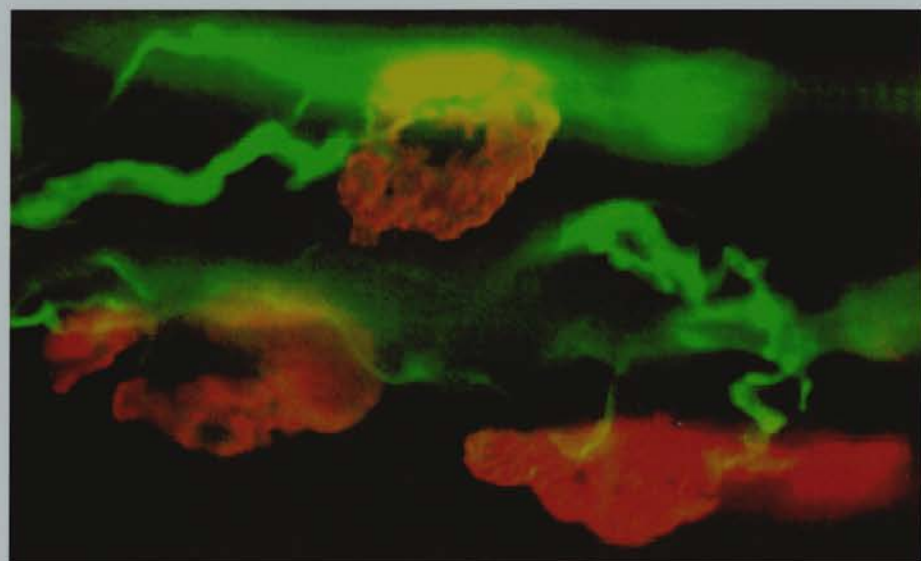


Fig 4.3 Partially occupied endplates were represented in all the 4DL muscles examined 3 days after LPN crush. Axons and nerve terminals were immunolabelled with FITC antibodies; AchRs were stained with TRITC- α -BTX. In all the muscles, there was a range of the fraction of the AchR plaques that was without innervation. (a): arrows indicate small regions of the receptor plaque with no covering nerve terminal boutons. However, SV2-stained boutons were apparent in the remainder of the junctions. (b): the arrow indicates only one small region of innervation over the AchR plaque. In this example, the bouton appears yellow because of the overlap between FITC and TRITC staining. The vast majority of this endplate is not occupied. Scale bar is 20 μ m.

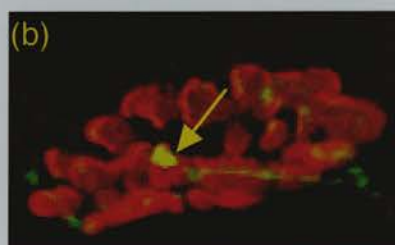
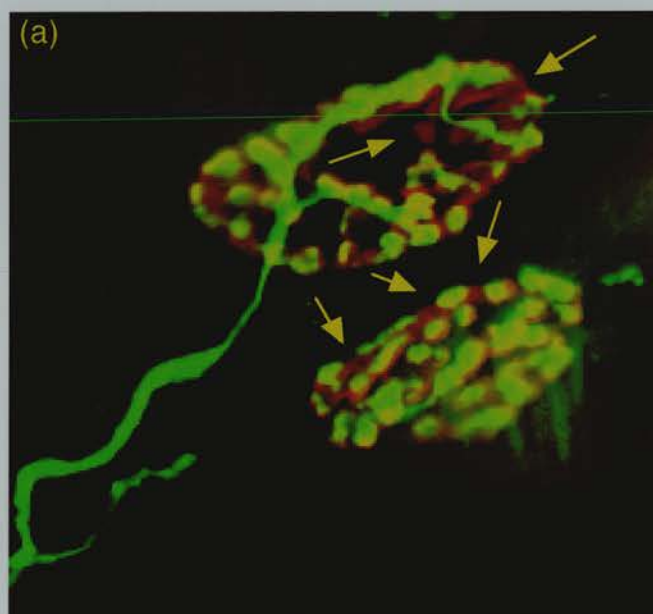
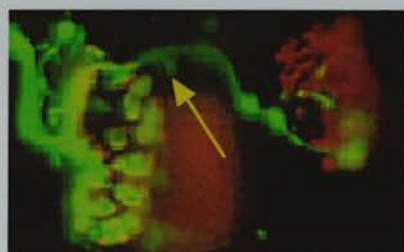
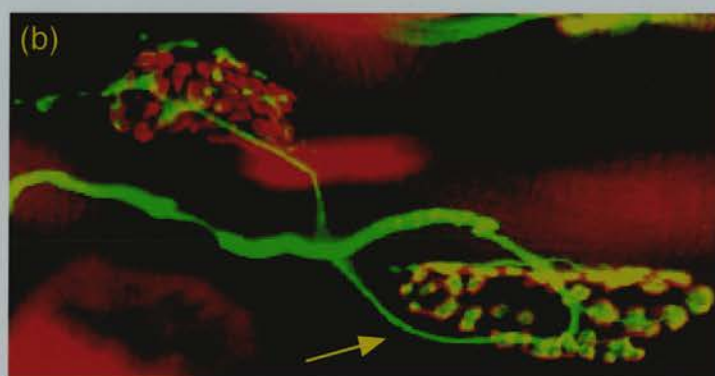
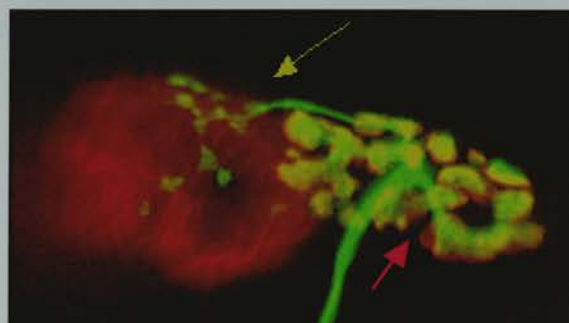
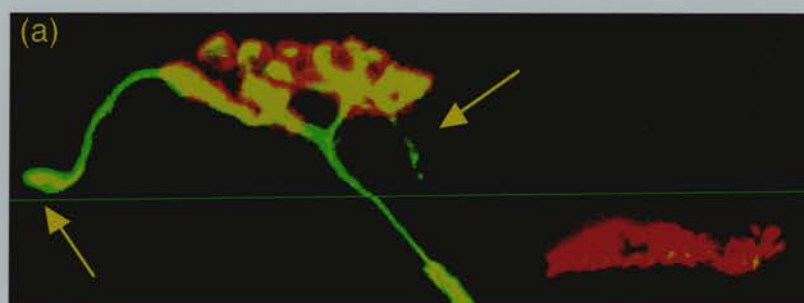


Fig 4.4 Nerve terminal sprouting was visible in 4DL muscles 3 days after LPN crush. Muscles were fixed and immunolabelled with FITC antibodies to nf/SV2 and AchRs were stained with TRITC- α -BTX. Yellow arrows indicate nerve sprouts; unlabelled axons are derived from parent collaterals. (a): The top examples show fully occupied endplates extending nerve terminal sprouts, each projecting toward nearby denervated endplates. Note the lack of nf and SV2 staining at the denervated endplates. The bottom examples show partially occupied endplates extending nerve sprouts toward nearby denervated endplates. (b): Both examples given show that in some cases, nerve sprouts contacted denervated endplates 3 days after LPN crush. Scale bar is 20 μ m.



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Fig 4.5 A few 4DL endplates were fully occupied, 3 days after LPN crush. Presynaptic structures were immunolabelled with FITC nf/SV2 and postsynaptic structures were stained with TRITC- α -BTX. (a): the top panel shows three examples of presynaptic staining alone; the bottom panel shows the configuration of AchRs at the endplates. All three endplates were fully occupied, indicated by the 100% correlation between the area of pre-and postsynaptic staining. (b): a fully occupied endplate, where 100% innervation is indicated by the overlap between FITC and TRITC staining. Scale bar is 20 μ m.

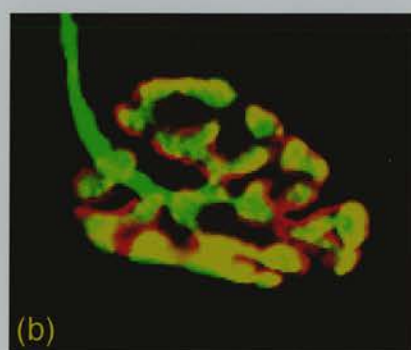
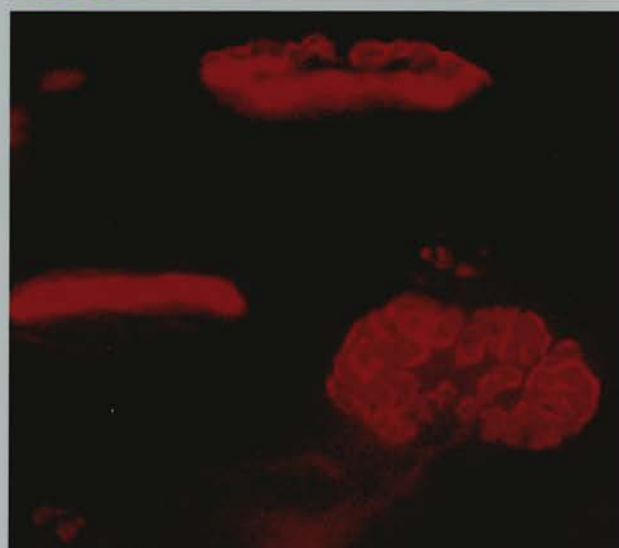
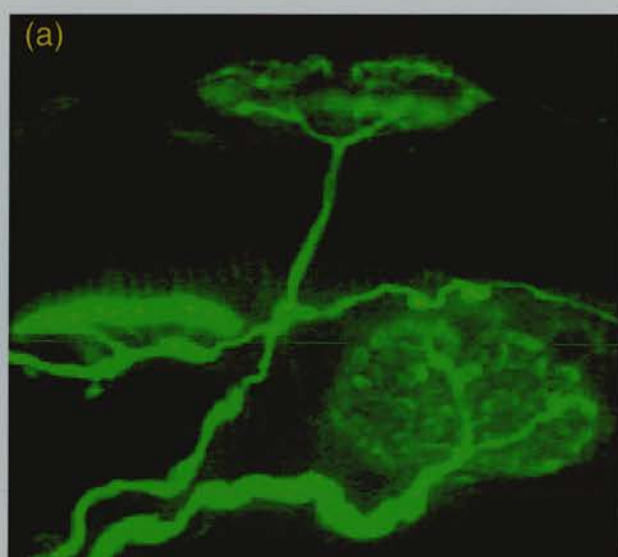


Fig 4.6 Patterns of polyneuronal innervation in 4DL muscles after recovery from LPN crush and TTX paralysis. Orange boutons indicate LPN staining (by RH414) and green boutons belong to SN axons (stained by FM1-43). In all muscles, there were examples of polyneuronal innervation where the SN contributed the larger fractional occupancy to the endplate (a), the LPN was the larger of the two inputs (b), and both nerves contributed approximately equally to endplate area (c). Scale bar is 20 μ m.

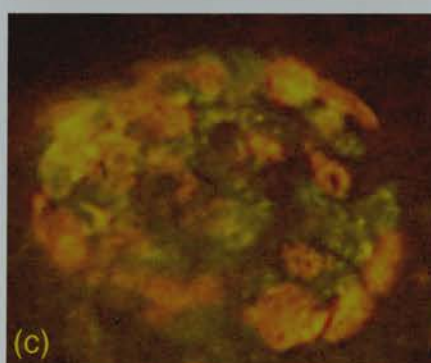
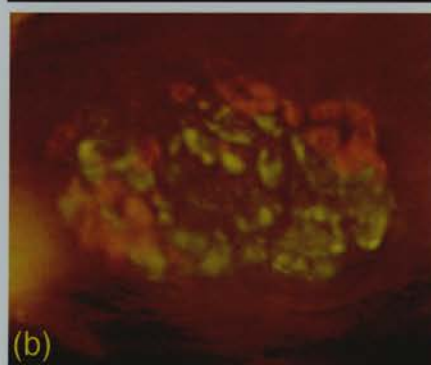
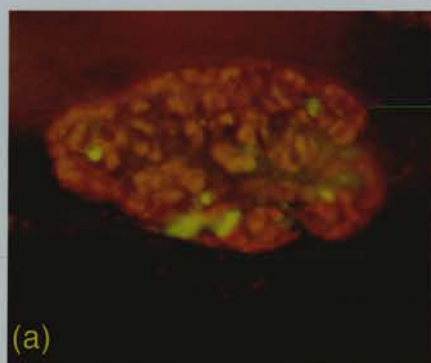


Fig 4.7 With the exception of innervation pattern, the morphology of 4DL endplates was similar throughout the experimental muscles. Top graph: small endplates were no more likely to be polyneuronally innervated than large endplates. The mean size of endplates for each pattern of innervation is given. Error bars are \pm standard deviation. A one-way ANOVA of the endplate sizes for each pattern of innervation was performed. There was no significant difference between the endplate sizes of polyneuronally innervated (N=3 muscles; n=13 endplates), SN mononeuronally innervated (N=3 muscles; n=23 endplates) or LPN mononeuronally innervated endplates (N=3 muscles; n=15 endplates) in the experimental muscles. Bottom graph: small endplates had fewer boutons per endplate than large endplates, only if they were mononeuronally innervated by the LPN (red circles). There were no significant correlations between endplate size and number of boutons per endplate at polyneuronal (yellow circles) or SN mononeuronally innervated (green circles) endplates.

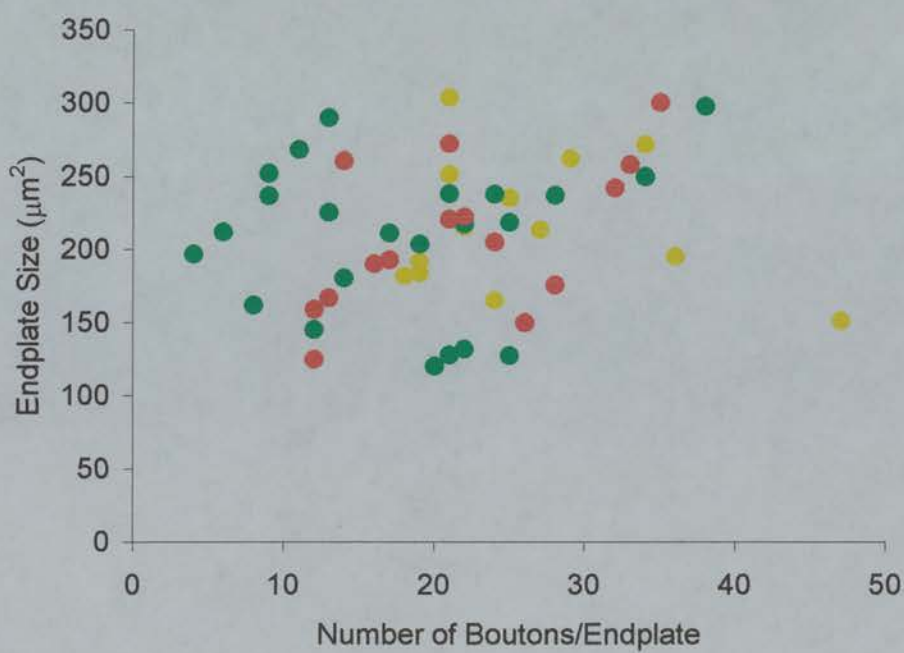
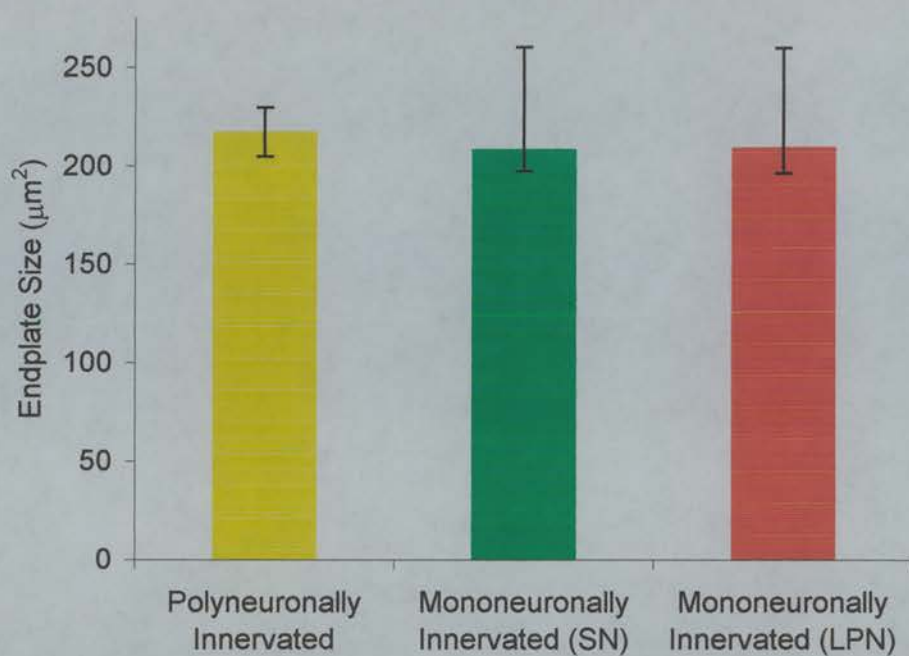
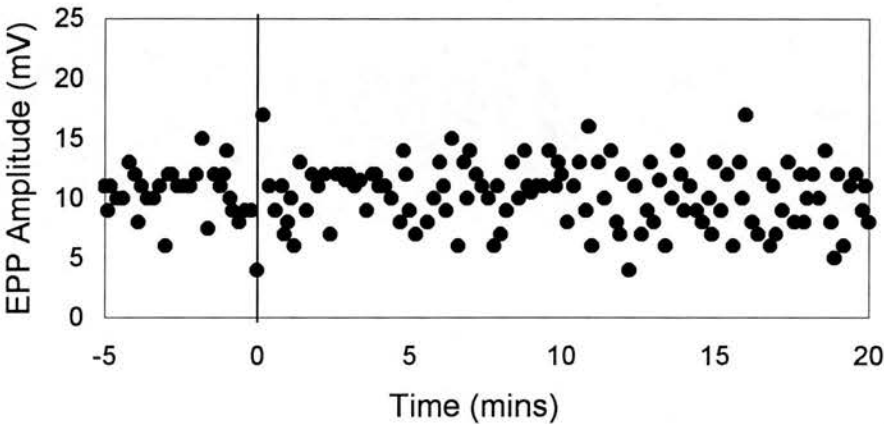


Fig 4.8 The styryl dyes did not affect subsequent synaptic transmission at 4DL neuromuscular junctions. A polyneuronally innervated muscle fibre was located using electrophysiology. The amplitude of LPN (a) and SN (b) EPPs were recorded every 10 seconds, and are expressed as individual dots in both graphs. After 5 minutes of recording the baseline amplitude of LPN EPPs, the nerve terminals were stained with RH414. The vertical line at time 0 indicates the dye loading. EPP amplitudes were recorded for a further 20 minutes. Then, the SN EPP baseline was recorded, nerve terminals were stained with FM1-43 and the EPP amplitude was recorded for a further 20 minutes.

(a) LPN



(b) SN

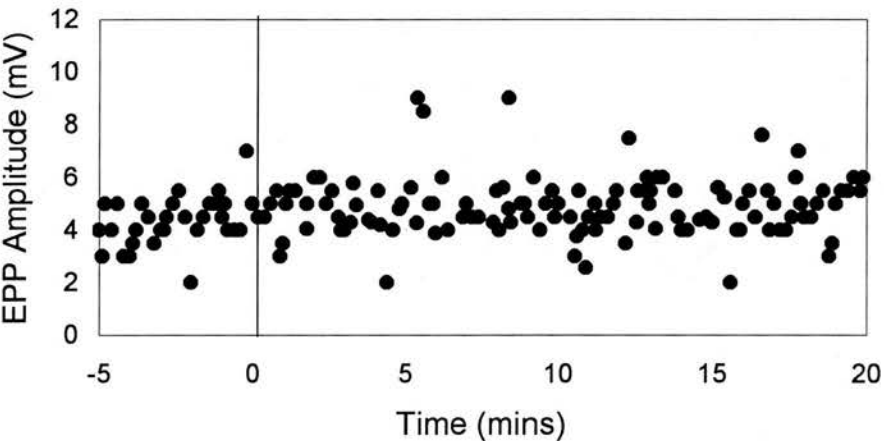


Fig 4.9 Morphology and electrophysiology of polyneuronally innervated 4DL endplates. FM1-43 and RH414 were used to label synaptic boutons supplied by SN and LPN motor axons in isolated preparations. A,B and C: composite digital images from three different neuromuscular junctions, with examples of intracellularly recorded EPPs evoked from the junctions after bathing in μ CTX to abolish muscle action potentials. In each case, the orange dot indicates LPN stimulation, and the yellow/green dot indicates SN stimulation. The relative amplitudes varied in proportion to the relative areas covered by LPN and SN synaptic boutons. Cyan scale bar is 30 μ m; black scale bars: 10msec/10mV (for x and y axis, respectively).

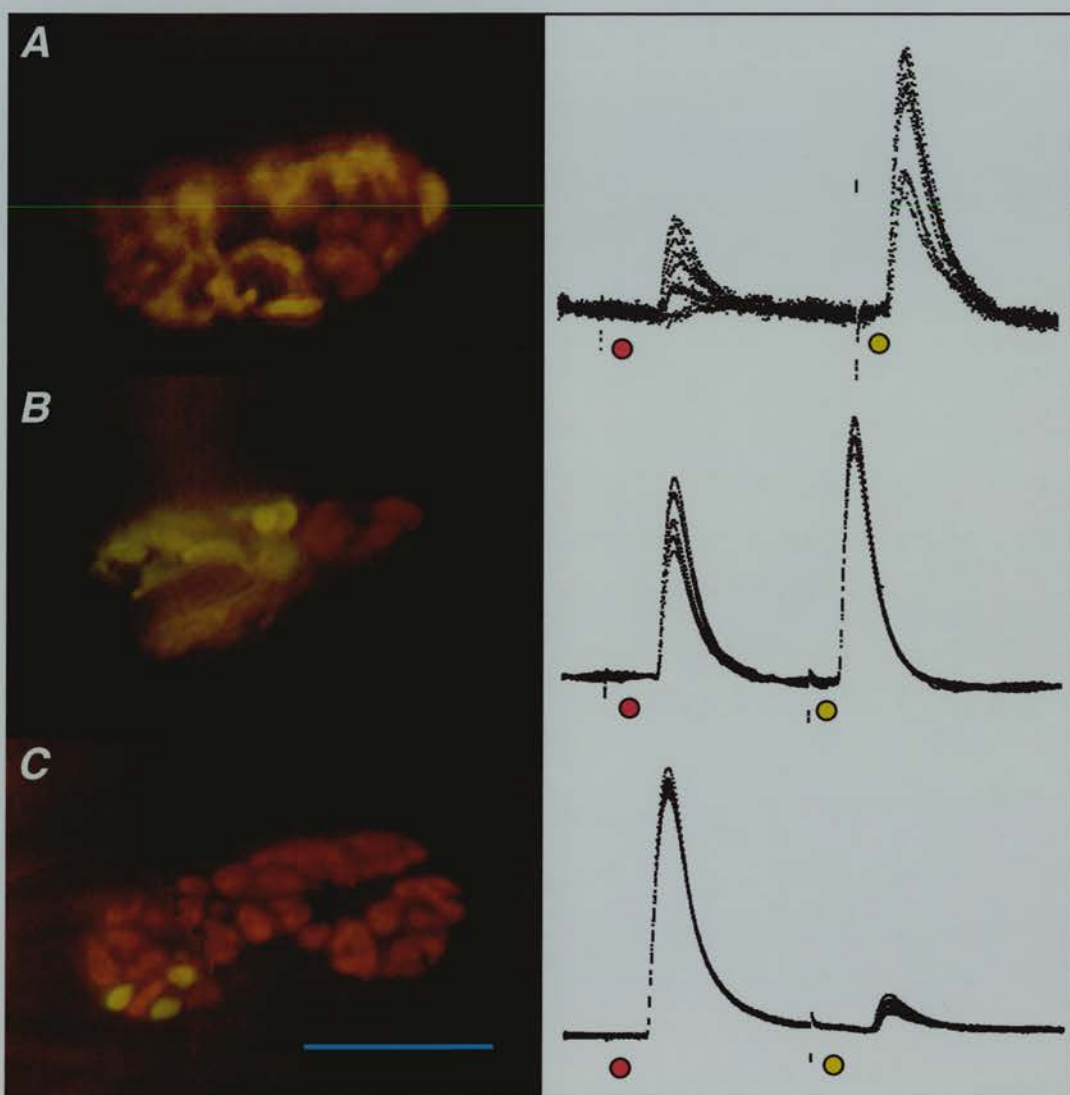


Fig 4.10 Small inputs were weaker than large inputs at polyneuronally innervated 4DL endplates (a & c). All LPN (a) and SN (c) inputs from 18 neuromuscular junctions were expressed in terms of their percentage contribution to total junctional size and effect. Occupancy (% of total) was calculated as follows:

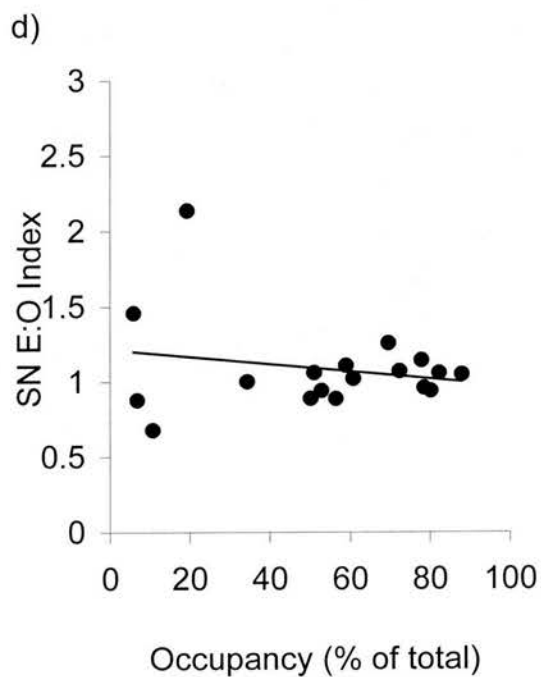
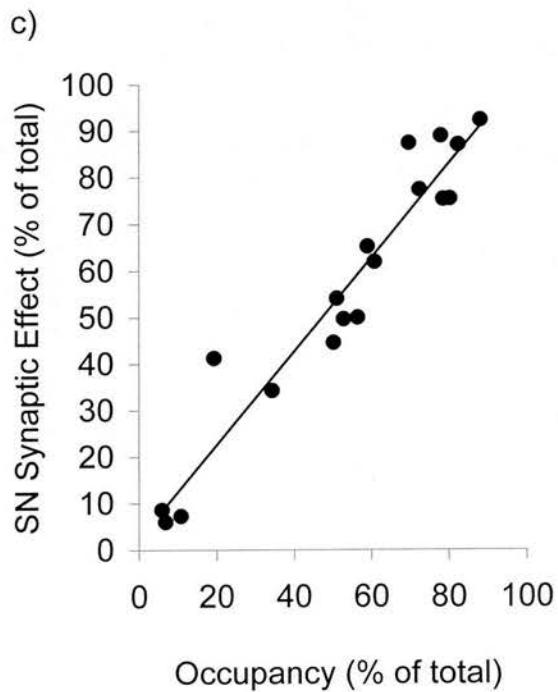
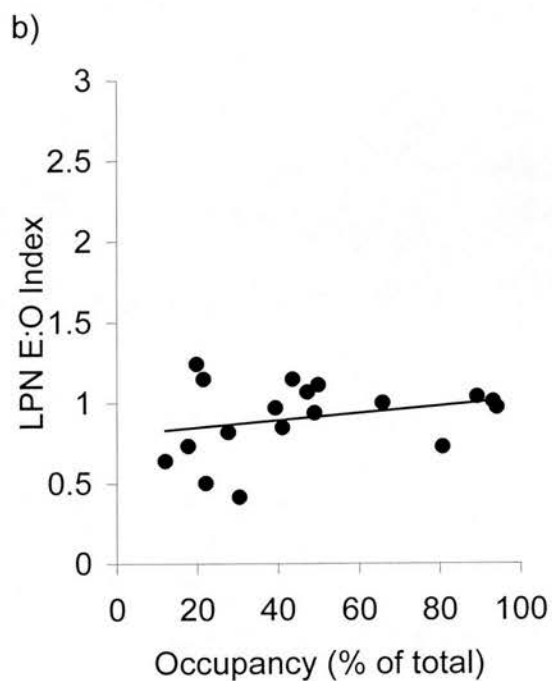
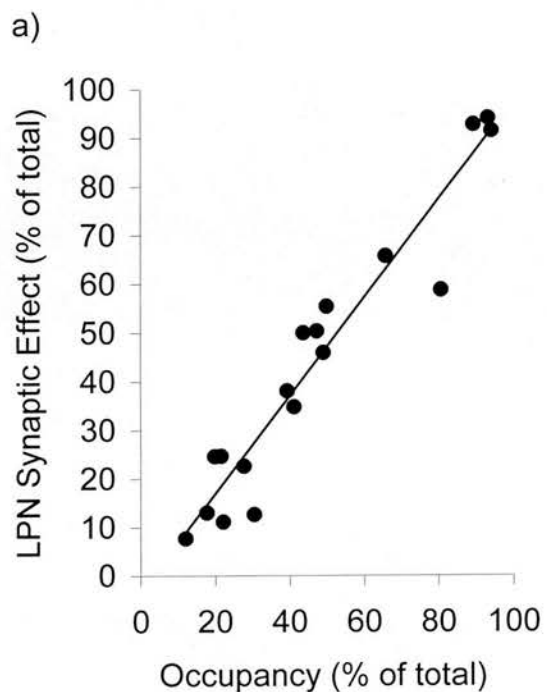
$$[\text{size of input}] / [\text{size of LPN input} + \text{size of SN input}] * (100)$$

where input size was measured in μm^2 . Synaptic effect (% of total) was calculated as follows:

$$[\text{EPP amplitude of input}] / [\text{LPN EPP amplitude} + \text{SN EPP amplitude}] * 100$$

where EPP amplitude was measured as the mean of 100 responses to nerve stimulation.

Small inputs were not disproportionately weaker than large inputs, when normalised for size (b & d). LPN (b) and SN (d) inputs from 18 neuromuscular junctions were expressed in terms of their contributions to total endplate occupancy vs. their e:o indices. Occupancy (% of total) was calculated as described above; see the Introduction to this chapter, section 4.3.3, for the derivation of the e:o index.



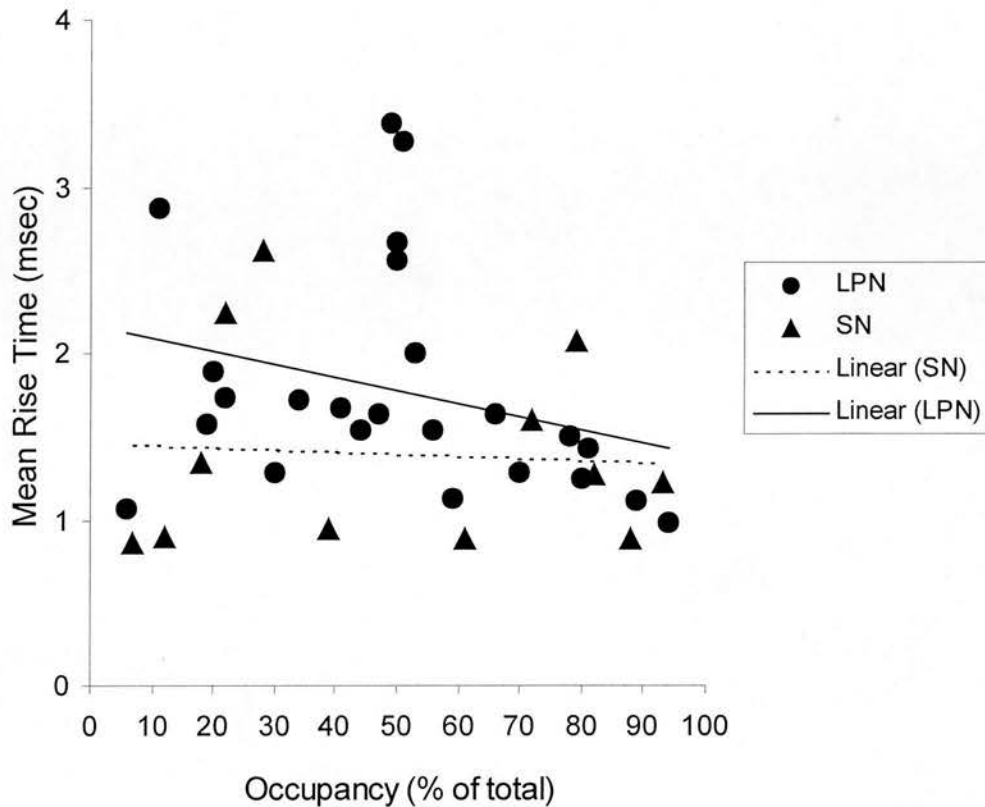


Fig 4.11 Small and large LPN and SN inputs to polyneuronally innervated 4DL endplates showed equivalent EPP rise times. The contribution to total endplate size of each input in the data set (two per endplate; one LPN and SN) is plotted against the input's mean rise time from 100 EPPs. The regression line through the LPN data indicates a slight negative correlation ($m=-0.01$; $r=-0.28$), but this was not significantly different from zero (tested at the 5% confidence limits for a Pearson correlation of 0.00). The regression line through the SN data showed neither a positive nor a negative correlation between the rise times of small and large inputs ($m=-0.001$; $r=-0.07$) which was not significantly different from a Pearson correlation of 0.00.

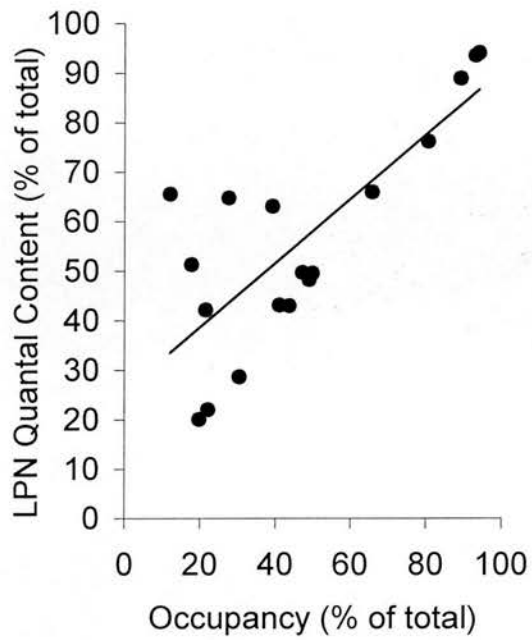
Fig 4.12 Small inputs released less neurotransmitter than large inputs at polyneuronally innervated 4DL endplates (a & c). All LPN (a) and SN (c) inputs from 18 neuromuscular junctions were expressed in terms of their percentage contributions to total junctional quantal content and size. Occupancy (% of total) was calculated as described in **Fig 4.10**. Quantal content (% of total) was calculated as follows:

$$[\text{QC of input}] / [\text{LPN QC} + \text{SN QC}] * 100$$

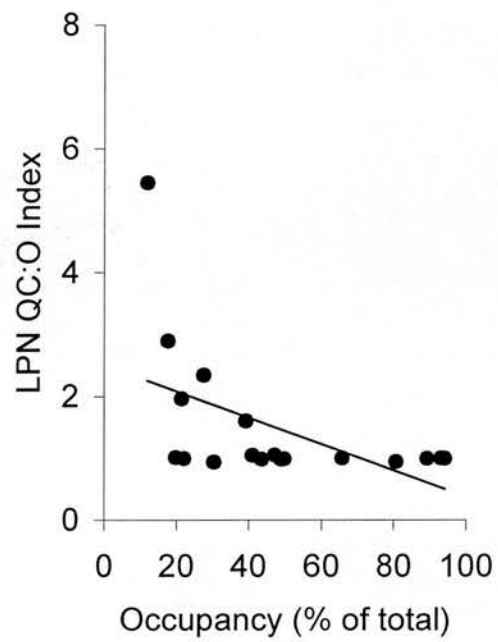
where QC was measured by the variance method (see Materials and Methods, section 2.4).

Small LPN and SN inputs released disproportionately less neurotransmitter than large inputs, when normalised for size (b & d). LPN (b) and SN (d) inputs from 18 neuromuscular junctions were expressed in terms of their contributions to total endplate occupancy vs. their qc:o indices. Occupancy (% of total) was calculated as described in **Fig 4.10**; see the Introduction to this chapter, section 4.4.2, for the derivation of the qc:o index.

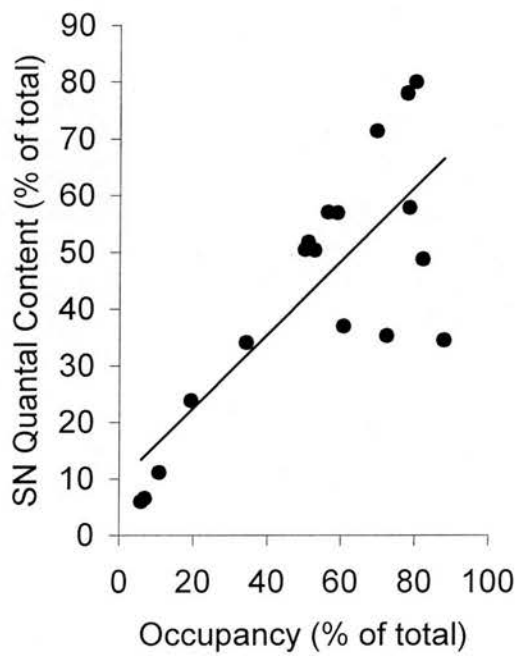
a)



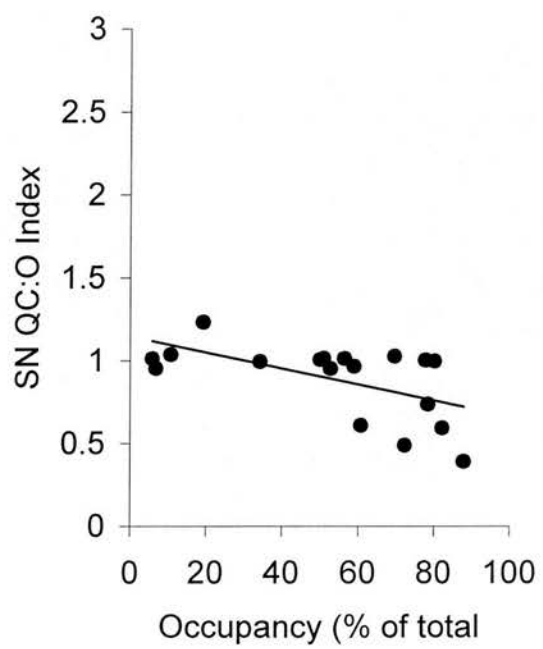
b)



c)



d)



MEPP	LPN	SN	p
amplitude (mV)	0.6 ± 0.04	0.8 ± 0.2	>0.05
frequency/second	2.8	2.3	>0.05

Table 4.1 There were no significant differences between MEPP characteristics from convergent inputs at polyneuronally innervated 4DL endplates. Polyneuronal endplates were determined using electrophysiology. The LPN and SN were tetanically stimulated in turn and MEPPs were collected (see the Introduction to this chapter, section 4.5.1). The mean (\pm s.e.m.) MEPP amplitude and frequency/second from LPN and SN inputs were compared at 6 polyneuronally innervated endplates (n=4 muscles). There were no significant differences between these measures (Mann Whitney t test).

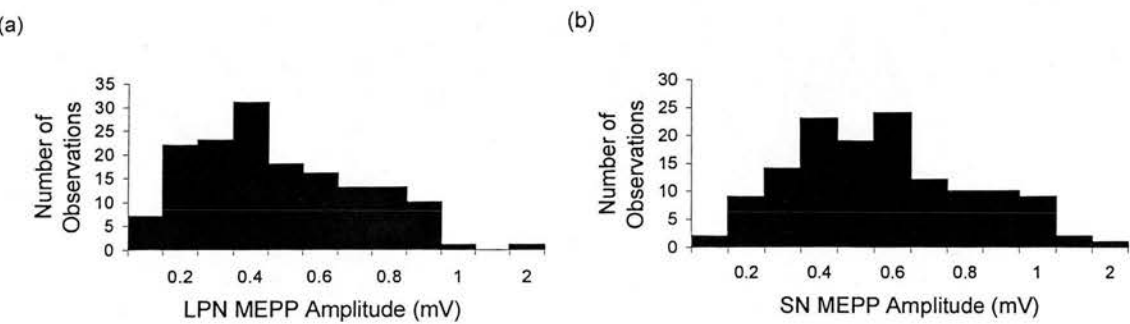


Fig 4.13 Amplitude distribution of spontaneous potentials from one polyneuronally innervated 4DL endplate. The histograms show the number of MEPPs from the LPN (a) and the SN (b) observed at each amplitude in a 30 second interval. The spontaneous potentials were not all the same size, but were distributed around the mean amplitude, in both cases.

Fig 4.14 The density of AchR clustering was qualitatively similar beneath large and small inputs at polyneuronally innervated 4DL endplates. LPN and SN inputs were stained with RH414 and FM1-43 (left hand panels), respectively, and polyneuronally innervated endplates were identified. Muscles were fixed, AchRs were labelled with TRITC- α -BTX, and endplates were relocated. Arrows indicate the location of small inputs (left hand panels) and the regions of the receptor plaques they innervate (right hand panels). In both examples, the region of the plaque occupied by the small input was no dimmer in fluorescence intensity than any other region of the receptor plaque. Scale bar is 15 μ m.

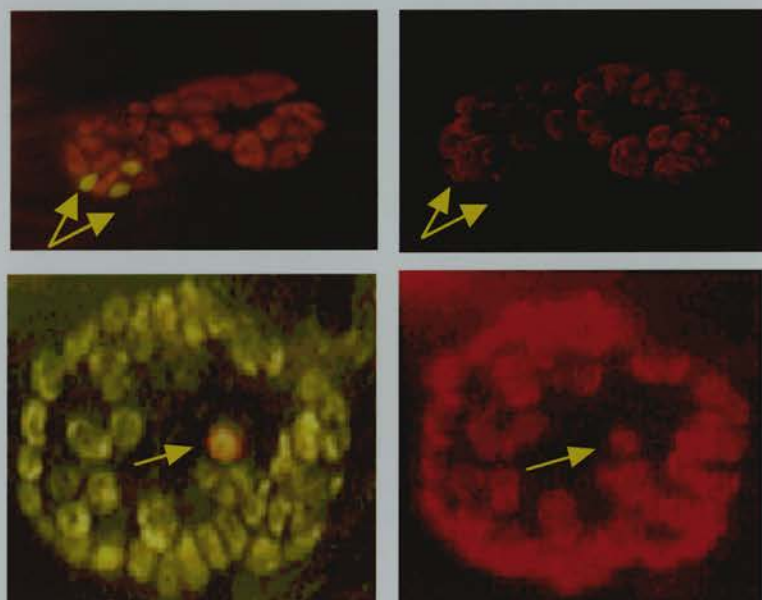
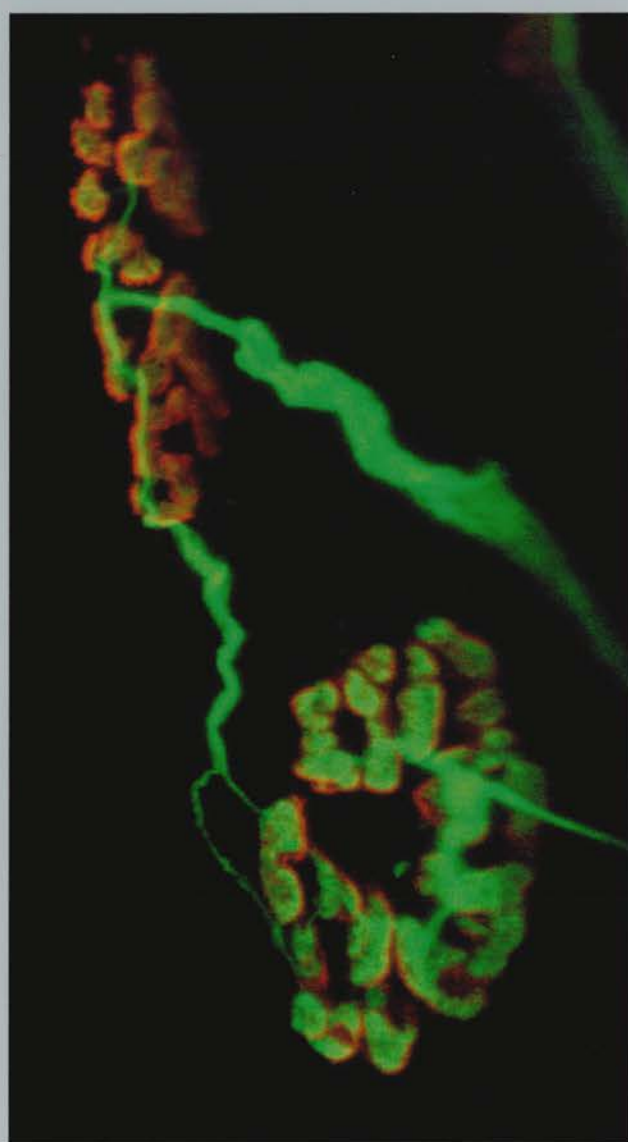


Fig 4.15 Polyneuronally innervated 4DL endplates can be distinguished by immuno-labelling. In this example, axons and synaptic vesicle proteins were FITC-immunolabelled and AchRs were stained with TRITC- α -BTX. At both endplates, the parent axon enters to provide innervation from the right hand side of the image. In addition to the parent axon, the lower endplate receives dual innervation from a nerve sprout derived from the upper endplate. The sprout diverges just before entering the endplate, a property which identifies the upper endplate as being responsible for projecting, rather than receiving the sprout. Scale bar is 10 μ m. *Image provided by Dr. Jacqueline Barry.*



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Discussion

In the experiments described in this chapter, the properties of persistently polyneuronally innervated endplates were examined by directly correlating the synaptic sizes and strengths of convergent inputs. The majority of inputs showed equivalent structure/function relationships, a finding which contrasts sharply to prevailing evidence about the mechanisms governing synapse elimination in skeletal muscle. The results suggest that (1) the temporary period of neuromuscular paralysis prevented subsequent synapse elimination (2) small LPN and SN inputs were not being eliminated from the endplates, and, therefore (3) polyneuronal innervation is not necessarily an unstable pattern of innervation.

4.11 Effects of Partial Denervation on 4DL Muscles

The first experiments in this chapter described the SN response to LPN denervation. That none of the muscles included in the analysis contracted to stimulation *in vitro* provided an indication that the method of partial denervation (LPN crush) was successful. The two muscles which also showed no SN contraction to *in vitro* stimulation were discarded from analysis because it was assumed that these muscles had never received innervation by SN axons. However, the proportion of muscles in the group that did not respond to SN stimulation was 25% (2 out of the 8 muscles that had been partially denervated by LPN crush), and this figure is consistent with the percentage of 4DLs that receive innervation from the LPN alone (Betz et al., 1979). Thus, it is unlikely that the SN failed to respond to LPN denervation in only these two muscles. The presence of an overwhelming majority of vacant and partially occupied endplates, together with the extent of nerve terminal sprouting suggests that the nerve crush was effective in removing LPN innervation from the muscles and triggering SN expansion.

4.12 Effects of Styryl Dyes on LPN and SN Transmission

It would be possible to explain the proportionate weakness of small inputs if the styryl dyes had had a normalising effect on the synaptic transmission of convergent axons. However, the results from the experiment in which EPP amplitudes were compared before, during and after staining inputs with FM1-43 and RH414 (see section 4.8.1) revealed no changes in the absolute or relative sizes of synaptic responses, suggesting that after washing from the bathing medium, the styryl dyes did not influence synaptic function. There have previously been reports of a pharmacological effect of RH414 on synaptic transmission (Grinvald et al., 1986). Bewick and Betz (1994) described an inhibition of EPP and MEPP amplitudes that could be reversed by exposing nerve-muscle preparations to fluorescent light from a mercury arc lamp. In their study, concentrations of RH414 between 5-42.5 μ M were effective in producing synaptic depression. In the control experiment described here (as in all the experiments where RH414 was used to stain LPN terminals), the concentration of RH414 was approximately 20 μ M. In contrast to Bewick & Betz's (1994) findings, there was no observed synaptic inhibition from either nerve during dye application (see Fig 4.8). The reason for this difference may have been that the control experiment was performed under illumination; Bewick & Betz reported disinhibition within 800 msec of exposing their preparations to light (this was found when the intensity of illumination was 10-100%).

Regardless of the discrepancy between current and previous findings, the results of the control experiment (see section 4.8.1) provide confidence that the obtained e:o

and qc:o indices were reflections of the synaptic function of the convergent inputs, rather than of an experimental manipulation. In addition, Bewick & Betz (1994) did report that the dye-induced inhibition was totally reversed by washing preparations in dye-free physiological saline for 30 minutes. In all the experiments reported here, muscles were washed between staining and electrophysiological recording for at least 30 minutes, which was probably sufficient to remove residual dye from the muscles and reverse any synaptic effects of RH414.

A related issue is that the high frequency stimulation (HFS) used to induce vesicle recycling (thereby labelling terminals) may have produced synaptic potentiations and/or depressions of the transmission at the convergent inputs. That is, stimulating the SN before the LPN to label nerve terminals may have resulted in a potentiation of transmission at the SN synapses and a depression of transmission at LPN synapses. This type of “heterosynaptic” depression has been described between embryonic *Xenopus* nerve-muscle synapses in culture (Dan & Poo, 1992, 1994): active terminals (Lo & Poo, 1991; Lo & Poo, 1994) or just the application of Ach from a pipette to the muscle fibre (Dan & Poo, 1992) can depress release from an inactive terminal innervating the same muscle fibre for an hour or longer. Similar results have been described *in vitro*, albeit on a shorter time course: Betz et al. (1989) showed that stimulating one input to a dually innervated neonatal rat 4DL endplate reduced transmitter release from the other input. The average depression was approximately 15%, compared to controls, but lasted for just tens of milliseconds. Both the magnitude and duration of depression increased with multiple conditioning stimuli. In contrast to the *Xenopus* experiments in culture,

Betz et al. did not attribute the observed depression to heterosynaptic effects of the convergent inputs; rather, they concluded that the depression was the result of changes in ion concentrations in the synaptic cleft, which led to reduced Ach release in response to stimulating the second input following stimulation of the first input. If similar mechanisms were triggered by the HFS during dye loading, any depressive effects should have been obvious during the control experiment. Therefore, although the control experiment described in this chapter was directed towards determining an effect of the styryl dyes on transmission, the results are also applicable to a refutation of the possibility that the HFS loading protocol differentially advantaged or disadvantaged LPN and SN inputs.

4.13 The Relationship Between Synaptic Size and Effect

The high correlations between fractional synaptic occupancy and effect (see Figs 4.10a & c and 4.12a & c) indicated that small LPN and SN inputs were weaker than large LPN and SN inputs. This relationship was not unexpected: synaptic strength is primarily a function of quantal content, which in turn depends on nerve terminal size. It has been shown that the postsynaptic response to a single quantum of transmitter is variable, but the mean amplitude decreases proportionately with increasing junctional size and muscle fibre diameter (Katz & Thesleff, 1957; Kuno et al., 1971), implying that the amount of transmitter released in a single quantum is relatively uniform. However, quantal size increases proportionately with junctional size/muscle fibre diameter (Wilkinson et al., 1992), implying that more quanta are released per synaptic impulse from larger inputs.

The strong correlations are consistent with the idea that the small inputs were being eliminated from endplates by their larger, stronger competitors (Balice-Gordon & Lichtman, 1993,1994; Colman et al., 1997). However, the *lack* of significant correlations between fractional synaptic occupancy and effect when inputs were normalised for size (see **Fig 4.10a & c** and **4.12b & d**) argues against this possibility. If the present results were consistent with the earlier reports from J.W. Lichtman's laboratory, the correlation between input size and e:o index should have been significantly positive: the smallest inputs would have had calculated e:o indices much less than 1.0, which increased with increasing fractional occupancy (the larger the input, the stronger the absolute synaptic effect).

The results also disagree with a report by Thompson et al. (1979). In this study, neuromuscular inactivity delayed synapse elimination, but unlike the present results, paralysis was not found to prevent the process irreversibly. Thompson et al. found that the loss of multiple innervation in the rat soleus muscle was almost completely blocked during a 9 day period of inactivity, but once the activity block was removed, synapse elimination occurred – outside the normal period of synapse elimination. The present results are clearly contradictory to these data. This contradiction suggests that the effects of paralysis on synapse elimination may be fundamentally different in the neonate and adult. However, it is important to note that the duration of activity block used here (2-10 weeks) was much longer than that used by Thompson et al. (1979). Thus, it may be that longer periods of paralysis are required in order to produce irreversible effects on synapse elimination. Consistent with this, Brown et al. (1982) found that BoTox blockade (which irreversibly blocks

transmitter release; Brooks, 1956) during neonatal synapse elimination led to persistent polyneuronal innervation after activity resumed. Likewise, in reinnervated muscle, Barry & Ribchester (1995) found persistent polyneuronal innervation after paralysing 4DL muscles for a minimum of 2 weeks.

The results do confirm and extend other reports of long-lasting polyneuronal innervation, which has been described under a variety of conditions. Analogous to the present results, all of these studies suggest that activity is not sufficient to trigger and/or maintain synapse elimination at all polyneuronal innervated muscle fibres.

4.13.1 Equivalent Structure:Function Relationships In Reinnervated Muscle

The results have the most similarity to those of Barry & Ribchester (1995), who determined the extent of synapse elimination in the 4DL following the resumption of activity after reinnervation and paralysis. In their experiments, muscles experienced some degree of synapse loss during the first 2 weeks of the resumption of activity, but thereafter there was no further loss, and approximately 30% of the muscle fibres retained polyneuronal innervation. In that study, the authors concluded that a period of paralysis generated an environment in which the inputs to some polyneuronal innervated endplates became consolidated, such that they were able to withstand subsequent forces of elimination (that is, the return of activity). However, there was no investigation of what the factors might be that conferred persistence on the endplates, or whether the patterns were truly persistent (see the Introduction to this chapter, section 4.1). The present experiments suggest that an appropriate relationship between the synaptic size and strength of inputs protects against

elimination. This type of protective relationship has been described before at frog neuromuscular junctions. Like mammalian junctions, frog muscles undergo a period of synapse elimination during the metamorphosis from tadpole to frog (Letinsky & Morrison-Graham, 1980). However, unlike mammalian junctions, the process slows before reaching completion, and approximately 14% of muscle fibres in adult frogs retain polyneuronal innervation (Herrera, 1984). Also unlike mammals, the polyneuronal innervation is usually presented in the muscle as mononeuronally innervated endplates at separate sites along the same muscle fibre, the distance between which represents between 10-30% of total fibre length (Diaz & Pecot-Dechavassine, 1988). Werle & Herrera (1988) denervated adult frog sartorius muscles, and compared the morphological and functional characteristics of polyneuronal innervated fibres before and after reinnervation. They found that a number of properties were unchanged. For example, the relative endplate potential sizes and nerve terminal lengths of the convergent inputs were the same before and after synapse elimination, suggesting that inputs were not eliminated on the basis of small size or efficacy. However, when synaptic efficacy was normalised for nerve terminal size, a strong correlation emerged: as in the present experiments, there was no difference between the strengths of inputs of different sizes.

One possible interpretation of these, and the present results is that inputs with inherently different release efficacies come to be randomly associated at polyneuronal innervated endplates during reinnervation. Synapse elimination then occurs selectively at those junctions where the two inputs differ the most, and polyneuronal innervation will persist at those junctions where the efficacies of the

two inputs are most similar. Following the lead of an early theoretical approach (Stent, 1973), it may be that competition between motor axons is governed by a Hebbian rule (Hebb, 1949), in which an important determinant of synaptic stability is the timing of pre- and postsynaptic activity. In mononeuronally innervated fibres, the different terminal branches of the same motor neuron may avoid mutual competition by virtue of their synchronous activity. However, in polyneuronally innervated fibres, it is likely that convergent axons would not be synchronously active; therefore, postsynaptic action potentials would pass beneath inactive presynaptic terminals some of the time. This pre-and postsynaptic asynchrony could destabilise both inputs, with the advantage going to the input better able to withstand the detrimental influence. The advantage may be determined by the release efficacy of an input, so that surplus axons are displaced from a junction only when in competition with other inputs of higher competitive strength. In contrast, competition between inputs of equivalent strength would lead to a stalemate in which polyneuronal innervation persists.

The above discussion touched briefly on the differences between synapse elimination in frogs and mammals. The most striking difference is that in frogs, embryonic polyneuronal innervation is not eliminated completely. However, there are additional differences: junctional growth in frogs is accompanied by substantial remodelling of pre- and postsynaptic structures (Herrera & Banner, 1987), and seasonal differences in the structure and function of frog junctions have been noted (Herrera, 1984). However, since there are also similarities in the overall structure and function of frog and mammalian junctions (Grinnell, 1995), much of the

plasticity may be explained by mechanisms of synapse elimination that are conserved across species. Given this homology, it may be argued that the present results do little to advance current knowledge about factors underlying stable polyneuronal innervation. However, in the Werle & Herrera (1988) study the nerve terminal lengths of convergent inputs were not measured as accurately as in the present study. The authors used a fluorescent dye to identify all of the innervation to a junction of interest, made recordings from several regions on the endplate, and then fixed and labelled the entire junction with silver staining. Using the different amplitudes of EPP responses the authors extrapolated which regions of the endplate were innervated by which nerve terminals, and thus estimated the sizes of convergent inputs. This technique is clearly not as accurate as using differently coloured styryl dyes to selectively label convergent inputs, and would result in a much larger margin of error. Thus, although the results from Werle & Herrera's study do agree with those presented here, the use of styryl dyes helps to support their earlier findings. In addition, the findings strengthen the position that mechanisms of synapse elimination may be common in whatever species the plasticity occurs.

4.14 Hormones

Several studies have shown that hormones can influence the rate and outcome of nervous system development (reviewed by Timiras, 1972). Only a few have been examined for their influence on neuromuscular plasticity and of these, the thyroid hormone has been the best described. This hormone has little effect on the synaptic properties of mature neuromuscular junctions (Hofmann & Denys, 1972; McArdle et al., 1977), but can profoundly influence neonatal development. For example, in a

study of hypothyroid rats, it was found that synapse elimination was slowed by about a week, while synapse elimination was accelerated in the muscles of hyperthyroid animals (Kawa & Obata, 1982).

Although the thyroid hormone probably acts similarly on all skeletal muscles, steroid hormones exert more specific effects. Postnatally, the androgen-sensitive levator ani muscle is normally only found in male rats (Cihak et al., 1970). Jordan et al. (1988) showed that synapse elimination is delayed in this muscle, lasting well into the second month after birth. However, if rats were castrated during the first postnatal week, synapse elimination was accelerated; if the castrated rats were treated with androgens, the loss was retarded (Jordan et al., 1989). Further, the level of polyneuronal innervation was sustained for at least 2 months after androgen treatment, suggesting that androgen treatment during neonatal development had permanent effects on the adult innervation patterns (Lubischer et al., 1992).

4.15 Distributed Polyneuronal Innervation

It has been suggested that physical separation of convergent inputs to a fibre is a means for mitigating competitive processes that would otherwise lead to the removal of all but one input. This type of distributed polyneuronal innervation is commonly described at frog neuromuscular junctions. As discussed above, frog muscles are characterised by multiple endplate sites distributed along the length of individual fibres. Isolated, innervated endplates on the same skeletal muscle fibre have also been described in mammals, although again, multiple sites do not normally persist unless separated by several millimetres (Brown et al., 1976; Kuffler, 1977, 1980).

Similarly, separation of convergent inputs appears to be an important factor ensuring the preservation of multiple innervation in autonomic ganglia (Hume & Purves, 1981), in which different axons innervate single neurons only if they can occupy different regions of the dendritic arbour (Purves & Hume, 1981; Hume & Purves, 1983; Forehand & Purves, 1984). Given these results, it might have been expected that the boutons supplying the polyneuronally innervated endplates in the present experiments would be separated by fairly large distances. This expectation was not supported by the data. Moreover, the degree of separation between convergent inputs on the 4DL fibres (usually only a few microns; see **Fig 4.6**) was much less than the amount of separation thought to be required ($>1000\mu\text{m}$) to maintain multiple innervation in skeletal muscle (Brown et al., 1976; Kuffler et al., 1977). Although separation of convergent inputs may well be an important mechanism for ensuring the long-term maintenance and stability of multiple innervation in some systems, it is clearly not a requirement in the 4DL muscle. Indeed, conclusions similar to the results reported here have been made about the spacing of persistent multiple inputs in adult skeletal muscle (Barry & Ribchester, 1995). Finally, even in the rabbit ciliary ganglion, the elimination of multiple inputs does not always seem to be influenced by proximity, even when competing inputs are located within the micrometer range (Forehand, 1987).

4.16 Possible Explanations for Persistent Polyneuronal Innervation

There are several possible mechanisms to explain the long-lasting effects of paralysis on synapse elimination. Since inactivity generally inhibits the time-course of synapse elimination, one possibility is that the polyneuronally innervated endplates

were functionally silent, *in vivo*. Although it has not been directly tested, this possibility seems extremely unlikely. Barry & Ribchester (1995) noted in their results that there were differences in muscle fibre input resistance in paralysed muscles versus controls, but no differences between muscle fibre input resistances 4 weeks after the resumption of activity. Since one of the earliest responses to muscle fibre disuse is a rise in input resistance (Berg & Hall, 1975), functionally silent polyneuronally innervated endplates should have been revealed on the basis of muscle fibre input resistances. Likewise, muscle contractile properties such as twitch time course and tetanus:twitch ratio recovered in 4DL muscles in parallel with behavioural signs of recovery from TTX block and muscle paralysis. If paralysis influences synapse elimination in the 4DL through an effect on activity patterns, it may be more likely that the result would be synchronised rather than blocked firing of the motor units. Indeed, findings from a number of studies suggest that synchronous activation may be a mechanism for maintaining convergent innervation (Hubel & Wiesel, 1965; Balice-Gordon & Lichtman, 1994), and approximately 12% of 4DL muscle fibres are electrically coupled at 3-5 days of age (Jones & Ridge, 1987), a time when muscle fibres are still polyneuronally innervated. Coupling between motor nerve terminals, at least, cannot be an adequate explanation for the present finding, however, because this would have made it impossible to selectively stain terminals at polyneuronally innervated endplates with FM1-43 and RH414. Motor axon coupling also fails to provide a satisfactory explanation. Each polyneuronally innervated endplate was innervated by a regenerating (LPN) and intact/sprouted (SN) terminal. These convergent inputs differed in their conduction velocities and response latencies (see Fig 4.9). Finally, it is unlikely that

synchronous activity in convergent inputs of uncoupled motor neurons could account for the persistent polyneuronal innervation, since it has been shown experimentally that the activity of all motor units to a muscle is not synchronised in the adult during voluntary movements (Hennig & Lomo, 1985). Thus, although I cannot rule out the possibility that persistent polyneuronal junctions were synchronously active, this seems to be the least parsimonious explanation for their existence.

It is also possible that paralysis influences synapse elimination through a mechanism unrelated to subsequent activity, possibly by permanently increasing the amount of muscle-supplied trophic substance for which motor terminals may compete (Purves, 1986). Consistent with this idea, a recent report described that both basic fibroblast growth factor and ciliary neurotrophic factor delayed synapse elimination in neonatal gastrocnemius muscles (English & Schwartz, 1995; also see the preceding Discussion, **Chapter 3**, for a consideration of neurotrophic influences on synapse elimination).

Although the actual mechanisms by which a period of paralysis prevents subsequent synapse elimination in the 4DL are not known, one general type of effect is possible. This assumes that synapse elimination can occur only during a critical period of development and after reinnervation, and that inactivity during this time blocks the process. By the time the period of inactivity has ended, the critical period for synapse elimination has passed, leaving the muscle with a higher than normal level of polyneuronal innervation. In this scenario, the action of paralysis is short-term, but the end result is permanent because paralysis operates throughout, and beyond,

the window of time during which synapse elimination can occur from a significant fraction of the neuromuscular junctions.

4.17 The Relationship Between Input Size and Quantal Content

The correlations between fractional synaptic occupancy and quantal content were not as strong as the correlations between fractional occupancy and overall synaptic effect (see **Figs 4.10** and **4.12**). One reason for this may have been that the small LPN and SN inputs released more neurotransmitter than large inputs, which would be consistent with the observed significant, negative correlations between normalised synaptic size and quantal content (see **Fig 4.12b & d**). This finding may be related to previous studies, which have shown that nerve terminals release increasing amounts of neurotransmitter when postsynaptic activity is decreased as the result of reduced receptor density. For example, Sandrock et al. (1997) recently reported that mice that were heterozygous for a null mutation of neuregulin had a reduction in the density of AchRs and, therefore, a reduction in quantal size. Neuregulins are a family of synaptically released substances that activate AchR synthesis and clustering (Jo et al., 1995; Yang et al., 1998). The neuregulin knockout mice exhibited a compensatory increase in the number of quanta released by the nerve terminals, caused not by an increase in terminal size, but by an increase in either the density of release sites or the probability of release from each site. However, transmitter replenishment mechanisms were unable to keep up with the abnormally high demand imposed on these terminals, and transmission failed upon repetitive activation. The observed transmission failure and resulting muscle weakness are similar to symptoms of the human neuromuscular disorder myasthenia gravis, where

quantal size is also decreased – in this case by the autoimmune destruction of AchRs – but there is a compensatory increase in the amount of transmitter released presynaptically (Cull-Candy et al., 1980). A similar upregulation of evoked transmitter release occurs in experimental myasthenia in rats, induced by blocking most of the AchRs by chronic infusion with α -BTX.

For the current results to be consistent with these reports, it follows that there should have been evidence that the receptors beneath the small inputs were reduced in density. This, in turn, would support the idea that the small inputs *were* in the process of being eliminated. However, no indication of weak receptors was found in the present experiments. First, a slowing of synaptic responses occurs when AchR density is low (Betz & Osborne, 1977; Kidokoro, 1980), presumably because of an increase in the time it takes for a neurotransmitter molecule to find an unoccupied receptor (Land et al., 1980). In contrast, in the present results, the rise times of the EPPs from small inputs were not significantly slower than those from larger inputs (see section 4.8.4). Second, differences in the fluorescence intensity of α -BTX can reveal portions of AchR plaques in adult muscles that are reduced in density (Balice-Gordon & Lichtman, 1994; Culican et al. 1988). No such differences in the intensity of fluorescence beneath small and large inputs were found in the present experiments (see section 4.10.2). These results indicate that the AchRs were uniformly distributed with equal density at the polyneuronally innervated endplates.

Thus, the reason for the elevated quantal contents at small inputs is unclear. However, it is not difficult to imagine a mechanism whereby decreased activity in

the postsynaptic cell could lead to an increase in presynaptic transmitter release. Such a retrograde signal would ensure a match between postsynaptic requirements for transmitter and presynaptic release characteristics so that the muscle fibres always receive adequate activation.

This type of regulation of transmitter release has recently been described in two studies of the *Drosophila* neuromuscular junction, where glutamate is the neurotransmitter. In these studies, flies were genetically manipulated so as to cause a selective reduction in quantal size. When either the density (Petersen et al., 1997) or efficacy (Davis et al., 1998) of postsynaptic glutamate receptors was reduced, there was a compensatory increase in the number of quanta released by the presynaptic terminals. As in the neuregulin-deficient mice, the increase was not associated with an increase in nerve terminal size or the addition of new nerve terminal boutons. Taken together, all these studies suggest the existence of a strong homeostatic mechanism for regulating transmitter release that is conserved across species. However, in the *Drosophila* studies, the opposite pattern of regulation of synaptic efficacy was not found, as would be expected in a true homeostatic system. When quantal size was increased by genetic manipulation of the glutamate receptors, there was no decrease in the amount of transmitter released (Petersen et al., 1997; Davis et al., 1998) and the muscle fibres were hyperactivated in the flies.

Thus, there does not appear to be a mechanism to reduce the amount of transmitter released at a synapse when quantal size is too large. Although such a mechanism would be useful throughout life to compensate for genetic or environmentally

induced alterations in synaptic efficacy, increases in quantal size are not normally a consequence of the developmental growth of muscle fibres or the return of activity following denervation. As a muscle grows (throughout development) and/or the input resistance drops (after reinnervation), a much larger synaptic current is required to depolarise the muscle and allow for efficient contraction (reviewed by Grinnell, 1995). Since developing and denervated muscle fibres require increasing amounts of transmitter, there may be no need for a mechanism to down-regulate quantal content in the face of increased postsynaptic activity.

The present results raise the possibility that there may be different signals regulating synaptic size and strength in reinnervated muscle. In normal development, size may be the primary variable regulated; that is, synapses may increase their strength exclusively by increasing their sizes. However, other mechanisms may be brought into play when size/strength mismatches occur later in life. Underscoring this possibility is the evidence that in none of the *Drosophila* studies where the experimental manipulation altered synaptic efficacy was there any compensatory change in synaptic size: it was only the transmitter release parameters that were altered. There may be a hierarchy in the types of compensatory responses, with some occurring only when others are no longer possible. For example, during development, neuromuscular junctions grow by expanding the size of the nerve terminal boutons in parallel with increasing muscle fibre diameter and length (Hopkins et al., 1985; Balice-Gordon & Lichtman, 1990). After reinnervation, muscle fibres recover some of the size that is lost as a consequence of denervation-induced atrophy, but there is little additional growth beyond the recovery of the

original size of the junctions (Bishop, 1982). If the transmitter requirements of the muscle fibre continue to be unmet, and the maximum size of the junction has been reached, new compensatory signals may need to be triggered in order to satisfy the activity demands of muscles.

There are still many questions to be answered. While muscle-derived diffusible signals such as arachidonic acid (Harish & Poo, 1992), nitric oxide (Ribera et al., 1998) and neurotrophic factors (Ribchester et al., 1998) have been shown to act on presynaptic terminals to enhance synaptic efficacy, the retrograde signal(s) used *in vivo* and the postsynaptic variable that is actually sensed to generate a retrograde signal are unknown. However, given the complexity that is already apparent, the neuromuscular junction – where both synaptic size and strength parameters can be directly correlated – will have distinct advantages for determining the cellular mechanisms that are responsible for synapse elimination.

4.18 Future Work

If activity is not sufficient to effect synapse elimination at all skeletal muscle fibres, other factors (alone or in conjunction with activity) must play a role in determining the outcome. One question that is raised by the results of the present study is whether there is something special about the muscle fibres that retain persistent polyneuronal innervation. For example, these fibres may secrete persistently high levels of a nerve trophic factor which encourages the retention of multiple axonal inputs. Glial cell line-derived neurotrophic factor (GDNF) may be a possibility for future investigation: it has recently been shown that in mice which were genetically

engineered to overexpress muscle-GDNF, skeletal fibres retained polyneuronal innervation for as long as GDNF levels remained high (Nguyen et al., 1998). GDNF can also mediate functional changes at the neuromuscular junction (Ribchester et al., 1998), making it a good candidate for explaining the persistent polyneuronal innervation and the structure: function relationships described here. GDNF is upregulated following denervation (Naveilhan et al., 1997), and it is possible that some muscle fibres express permanently high levels in the 4DL after reinnervation. *In situ* hybridisation for GDNF expression on 4DL polyneuronal innervated muscle fibres would be one method to investigate this possibility. In addition, visualising the GDNF receptors with EM may reveal that the receptor is preferentially located on axons supplying multiply innervated endplates.

Another possibility for future work raised by the present results concerns the effects of differential activity at polyneuronal innervated endplates. If, as the study suggests, differences in the timing of AchR activation do not necessarily lead to the loss of all but one input, it should be possible to reproduce this effect using other experimental paradigms. It may be feasible to repeat Balice-Gordon & Lichtman's (1994) experiments using the sternomastoid muscle, but to desynchronise activity at individual endplates by focally applying Ach to portions to the AchR plaque. In contrast to Balice-Gordon & Lichtman's method, this would produce elevated activity in regions of a receptor plaque against a background of "normal" activity elsewhere in the junction.

Finally, the role of the Schwann cell was not investigated in the present study, and this offers an interesting subject for future work. Following reinnervation, terminal Schwann cells become mitotic (Love & Thompson, 1998) and it may be that a higher than average number of terminal Schwann cells is associated with the persistent polyneuronally innervated endplates. Since the function of terminal Schwann cells is to insulate nerve terminal boutons, and these cells produce N-CAM (Jessen & Mirsky, 1984; Noble et al., 1985; Mirsky et al., 1986), it may be that Schwann cells can override activity-dependent synapse elimination signals in some cases. The availability of antibodies directed against Schwann cells for immunolabelling means this possibility could be easily pursued.

Chapter 5:
Structure:Function Relationships During Synapse Elimination:
Is There Evidence for a Critical Period?

5.1 Introduction

The results of **Chapter 4** indicated that the synaptic strengths of large and small inputs were proportionate, at polyneuronally innervated endplates. However, what remains unclear is whether this relationship plays a role in protecting small inputs from elimination. It is possible that after reinnervation and recovery from paralysis, all inputs – whether destined to be eliminated or not – have e:o and qc:o indices within the normal range. Thus, the finding that most of the persistent inputs had normal synaptic strengths may have been only coincidental, and the persistence of convergently innervated fibres may be entirely due to other mechanisms.

The preceding discussion introduced the possibility that a critical period for synapse elimination operates in skeletal muscle. If synapse elimination is governed by mechanisms that operate during a critical period, the structure: function relationships of convergent inputs would not necessarily contribute to their preservation. The experiments described in this chapter were directed towards an understanding of the extent to which normal synaptic strength is associated with protecting inputs from being eliminated in the two weeks following the resumption of activity in reinnervated, paralysed 4DL muscles. To address this, muscles were examined 0, 7 and 14 days after recovery from paralysis to ask whether the synaptic strength of small inputs was proportionate throughout the period of synapse elimination. There were two possible outcomes:

- 1) all inputs would have normal synaptic strength throughout the period, or
- 2) there would be more inputs with normal synaptic strength at the end of the recovery period than at the beginning.

The implications of each of these possibilities, respectively, are as follows:

- 1) An appropriate structure:function relationship does not confer a competitive advantage on inputs. This would imply a critical period for synapse elimination.
- 2) Synaptic strength does select inputs for preservation. This outcome would not preclude a critical period, but would suggest that inputs with normal strength are favoured.

5.2 Experimental Design

The paradigm used to produce polyneuronally innervated endplates was identical to that described in the previous chapter. To study the structure:function relationships during synapse elimination, animals were sacrificed 0, 7, or 14 days after recovery from TTX paralysis, judged by the return of behavioural indicators.

5.3 Morphological Changes During Synapse Elimination

In preliminary experiments, an investigation into changes in innervation patterns during the two week period was conducted. Nerve-muscle preparations were made from animals sacrificed 0,7, and 14 days after recovery from paralysis and LPN/SN inputs were stained with FM1-43/RH414, as described previously (see section 2.1.6). Muscles were placed on a glass slide (76x26mm, 1.0/1.2mm thick; Blue Star Micro Slides), cover-slipped (2mm diameter, No.1 thickness; BDH) and viewed under fluorescence illumination. Innervation patterns were scored and divided into 4 categories:

- 1) LPN mononeuronally innervated,

- 2) SN mononeuronally innervated,
- 3) polyneuronally innervated, where the LPN and SN supplied equal amounts of the endplate. Endplates where each nerve occupied 45-55% of total area were included in this category. This group will be referred to as “50/50 polys”.
- 4) Polyneuronally innervated where the LPN and SN supplied divergent amounts of the endplate. Endplates where one nerve occupied 1-44% and the other nerve supplied 56-99% of total area were included in this category. This group will be referred to as “divergent polys”.

The group of divergent polys was further subdivided into 2 categories:

- a) LPN dominant/SN subordinate, where the LPN innervated 56% or more of the endplate, and
- b) SN dominant/LPN subordinate, where the SN innervated 56% or more of the endplate.

5.4 Synaptic Function of Convergent Inputs With Varying Recovery Times From Paralysis

In order to assess the structure: function relationship, intracellular recordings were made from identified polyneuronally innervated junctions 0, 7, and 14 days after the resumption of activity. For all three time points, the relationship between the absolute synaptic size and effect of the convergent inputs was assessed, as described previously (see section 4.3.2.). Next, the small and large inputs were normalised for size and the relationship between relative synaptic size and effect was measured (using the equation for the e:o index; see section 4.3.3.). Finally, the relationship between synaptic size and the absolute and relative quantal contents of the

convergent inputs was determined (using the equation given in the previous chapter for the qc:o index; section 4.4.2.).

Results

5.5 Morphology of 4DL Neuromuscular Junctions During Recovery From TTX Activity Block (0, 7 and 14 Days)

Muscles in the 0 day recovery group were noticeably atrophic in appearance (n=8). Zero days after the resumption of activity, muscles twitched spontaneously when placed in normal oxygenated Ringers after dissection. In the 7 and 14 day groups, muscles appeared qualitatively to be no different in size to controls (n=12 and n=9 muscles, respectively); the *in vitro* spontaneous twitching had disappeared by seven days, and continued to be absent at day fourteen.

The total mean area of innervation (total size of endplates) at polyneuronally innervated junctions did not change significantly over the two week period ($P>0.05$, one way ANOVA; Fig 5.1a). However, the organisation of polyneuronally innervated endplates was visibly different at days 0, 7 and 14 (Fig 5.2). Quantitative analysis confirmed that the mean size of boutons increased during the two week recovery period ($P<0.01$, one way ANOVA; Fig 5.1b). At 0 days, the mean bouton size was $2.0 \pm 0.4 \mu\text{m}^2$; this increased nonsignificantly at 7 days (to $2.7 \pm 0.4 \mu\text{m}^2$; $P>0.05$), and increased significantly by day 14 (to $4.9 \pm 0.6 \mu\text{m}^2$; 0 vs. 14 days: $P<0.01$; 7 vs. 14 days: $P<0.05$).

The mean number of boutons per endplate decreased significantly during the period of recovery ($P<0.01$, one way ANOVA; Fig 5.1c). Between 0 days (when the mean number of boutons per endplate was 47 ± 2.8) and 7 days (when the mean number was 39 ± 2.7), the decrease was nonsignificant ($P>0.05$). However, the decrease by day 14

was significant (the mean number of boutons per endplate at day 14 was 28 ± 2.4 ; 0 vs. 14 days: $P < 0.001$; 7 vs. 14 days: $P < 0.05$).

Some of the innervation patterns in 4DL muscles also underwent changes during the two week recovery period (Fig 5.3; Table 5.1). First, the incidence of LPN mononeuronally innervated endplates increased, however, the observed rise was only significant 14 days after recovery (Fig 5.3; Table 5.1). Second, the levels of polyneuronally innervated endplates fell significantly between day 0 and 14 ($P < 0.05$, one way ANOVA). At 0 days, $52 \pm 6.3\%$ of the fibres examined were polyneuronally innervated. At 7 days, the level fell to $39.0 \pm 7.3\%$, although the decrease was nonsignificant ($P > 0.05$). However, at 14 days, $27.4 \pm 3.2\%$ of fibres were polyneuronally innervated, and the decrease was significantly greater compared to levels at day 0 ($P < 0.05$).

Polyneuronally innervated junctions were classified into two groups: those which were equivalently innervated by the LPN and SN (50/50 polys; see Fig 5.2b, left hand panel), and those which were innervated by unequal amounts of LPN and SN (divergent polys). In the group of divergent polys, sometimes the LPN was the major nerve supply (as in Fig 5.2c, right hand panel) and sometimes the SN was the dominant input (see Fig 5.2c, left hand panel). Between 0 and 7 days recovery, there were no significant changes in the percentage of muscle fibres in the divergent or 50/50 poly groups (Fig 5.3; Table 5.1). However, between days 7 and 14, there was a significant reduction in the percentage of 50/50 polys detected in the muscles (Fig 5.3; Table 5.1). There continued

to be no change in the percentage of divergent polys, between 7 and 14 days recovery (Fig 5.3; Table 5.1).

Finally, there was no significant change in the levels of SN mononeuronally innervated endplates between 0, 7 and 14 days recovery (Fig 5.3; Table 5.1).

When the group of divergent polys was subdivided, no significant changes in the levels of SN dominant/LPN subordinate polys were detected during the recovery period ($P>0.05$, one way ANOVA; Fig 5.4). However, levels of LPN dominant/SN subordinate polys in the group changed significantly ($P<0.01$, one way ANOVA; Fig 5.4). Between days 0 and 7 there was a significant increase ($P<0.01$), from $2 \pm 0.4\%$ of the group at day 0, to $5.6 \pm 0.8\%$ at day 7). The level declined significantly ($P<0.05$) by day 14, to $2.3 \pm 0.7\%$. There was no significant difference between the percentage of divergent polys where the LPN was the major nerve supply at days 0 and 14 ($P>0.05$).

5.6 Synaptic Function of Convergent Inputs at Polyneuronally Innervated 4DL Neuromuscular Junctions

5.6.1 Relationship Between Synaptic Size and Strength With No Recovery From TTX Activity Block

Of the neuromuscular junctions included in analysis ($n=4$ junctions; $n=8$ inputs), two were innervated by unequal amounts of LPN and SN boutons. In one junction, the LPN was the larger input, and in the other, the SN innervated the majority of the endplate. Intracellular recording revealed that stimulating the larger input produced a large synaptic response, and the smaller input produced a smaller response (Fig 5.5a). This

was found at both fibres. The remaining two junctions included in analysis were innervated by approximately equivalent areas of LPN and SN boutons. The EPP responses to nerve stimulation were approximately equal in amplitude, from both of these fibres (Fig 5.5b).

Fractional Occupancy: Fractional Synaptic Effect

Small LPN and SN inputs produced smaller synaptic effects than large inputs at polyneuronally innervated endplates (Fig 5.6a). There was a very strong correlation between the fractional occupancy and fractional synaptic effect of the LPN and SN inputs (Pearson's $r=0.99$; Fig 5.6a). The 95% confidence limits (0.73 to 0.99) indicated that the correlations were highly significant ($P<0.01$). Variance analysis indicated that the data was tightly clustered around the regression line: the mean difference between each LPN and SN input's contribution to total synaptic effect and the regression line was 1.6% ($\pm 0.66\%$).

E:O Index

There were also strong correlations between fractional occupancy and synaptic effects when LPN and SN inputs were normalised for size (LPN: Pearson's $r=0.96$; SN: Pearson's $r=0.98$; Fig 5.6b). The 95% confidence limits (LPN 0.05 to 0.10; SN 0.27 to 0.10) indicated that the correlations were highly significant ($P<0.05$). Variance analysis also revealed that the data was tightly clustered around the regression lines. The mean difference between each LPN input's e:o index and the regression line was 0.03 (\pm

0.01); the mean difference between each SN input's e:o index and the regression line was 0.04 (± 0.01).

Fractional Occupancy:Fractional Quantal Content

There were weak negative correlations between the fractional occupancy of LPN or SN inputs and their contributions to the total junctional quantal content (Pearson's $r=-0.65$ for both data sets; Fig 5.6c). However, the 95% confidence limits (LPN -0.99 to 0.83; SN -0.10 to 0.52) indicated that the relationship was not significantly different from zero ($P>0.05$): small and large inputs were just as likely to contribute the majority of total junctional quantal content as the minority. For example, the smallest input in the LPN data set (33% of total junctional size) showed the *highest* contribution to its total junctional quantal content (69.5%), but the second smallest input in the LPN data set (43% of total size) showed the second *lowest* quantal content contribution (only 39% of total). Likewise, the largest LPN input (66% of total) provided the *lowest* contribution to its total junctional quantal content (30.5% of total) and the second largest LPN input showed the second *highest* quantal content (57% and 61% of total junctional size and quantal content, respectively). Variance analysis showed that there was some scatter in the data, with several points falling a distance from the regression line. The mean difference between each LPN and SN input's contribution to total junctional quantal content and the regression line was 6.1% ($\pm 1.3\%$). However, much of this difference was due to one polynuronally innervated endplate, in which the SN input contributed disproportionately more (61% of total) and the LPN input contributed disproportionately

less (39%) to the total amount of transmitter released than was expected, based on their sizes.

QC:O Index

Finally, when LPN and SN inputs were normalised for size, there were no significant correlations between fractional occupancy and quantal content in either data set (LPN Pearson's $r=-0.88$ and confidence limits -0.10 to 0.83 , $P>0.05$; SN Pearson's $r=-0.93$ and confidence limits -0.10 to 0.27 , $P>0.05$; Fig 5.6d). Variance analysis revealed that overall, there was minimal scatter in the data. The mean difference between LPN qc:o indices and the regression line was $0.11 (\pm 0.02)$; the mean difference for the SN data set was $0.16 (\pm 0.04)$.

5.6.2 Relationship Between Synaptic Size and Strength After 7 Days of Recovery From TTX Activity Block

Eight neuromuscular junctions were included in analysis ($n=16$ inputs). Two junctions in the data set were innervated by approximately equivalent amounts of LPN/SN boutons. In both of these junctions, the EPP responses to nerve stimulation were approximately equal in amplitude. Six junctions were innervated by unequal amounts of LPN and SN boutons. In only one of these junctions did intracellular recording reveal a proportional difference between the synaptic sizes and effects of the convergent inputs. In the other five junctions innervated by unequal amounts of LPN and SN boutons, intracellular recording showed that stimulating the smaller input elicited either (a) a

disproportionately large EPP (three junctions), or (b) a disproportionately small EPP (two junctions).

Fractional Occupancy: Fractional Synaptic Effect

There were weak correlations between the fraction of a junction occupied by LPN and SN inputs and their contributions to total synaptic effect (Pearson's r for LPN and SN inputs=0.70; Fig 5.7a), which were not quite significant (*confidence limits for both data sets: -0.01 to 0.94; P=0.06*). There was more scatter in the data at day 7 than day 0: variance analysis indicated that the mean difference between each LPN and SN input's contribution to total synaptic effect and the regression line was 14.5% ($\pm 4.0\%$). Four of the sixteen inputs fell outwith this mean, reflecting two polyneuronally innervated endplates, each with one input that was stronger than expected, and one that was weaker than expected, based on their sizes.

E:O Index

When LPN and SN inputs were normalised for size, there were no significant correlations between the size of an input and its synaptic effect (*LPN data set: Pearson's $r=-0.38$, confidence limits=-0.86 to 0.44, $P>0.05$; SN data set: Pearson's $r=0.33$, confidence limits=-0.49 to 0.84, $P>0.05$; Fig 5.7b*). Again, there was more scatter in the data at day 7 than at day 0 for this parameter of synaptic structure: function. The variance analysis showed that the mean difference between each LPN input's e:o index

and the regression line was $0.41 (\pm 0.09)$; the mean difference between the SN inputs and the regression line was $0.32 (\pm 0.05)$.

Fractional Occupancy:Fractional Quantal Content

The correlation between fractional occupancy and fractional quantal content for LPN and SN inputs was also very strong (Pearson's $r = 0.83$; Fig 5.7c) and very significant (*LPN confidence limits*: 0.23 to 0.97, $P < 0.05$; *SN confidence limits*: 0.30 to 0.97, $P < 0.05$). There was less variance in the distribution of data for quantal content than for overall synaptic effect at day 7 (see above): the mean difference between each LPN and SN input's contribution to total junctional size and the regression line was 8.9% ($\pm 3.3\%$). Six of the inputs fell outside this mean difference; three were weaker than expected, based on their sizes (providing 31, 58 and 59% of total endplate size, but only 18, 33 and 42% of total synaptic effect, respectively) and three were stronger than expected (providing 40, 41 and 68% of total synaptic area, but 57, 67 and 82% of total synaptic effect).

QC:O Index

When LPN and SN inputs were normalised for size, the strong correlations between fractional occupancy and quantal content were no longer apparent (Pearson's r for LPN inputs = -0.04; Pearson's r for SN inputs = -0.27; Fig 5.7d) and were not significantly different from zero (*LPN confidence limits*: -0.69 to 0.72, $P > 0.05$; *SN confidence limits*: -0.82 to 0.53, $P > 0.05$). There was little scatter in the qc:o indices at day 7, with most of

the inputs showing qc:o indices that were clustered around the regression line (*mean difference LPN data set: 0.21 ± 0.08 ; mean difference SN data set: 0.18 ± 0.06*).

5.6.3 Relationship Between Synaptic Size and Strength After 14 Days of Recovery From TTX Activity Block

Seven neuromuscular junctions were included in analysis (n=14 inputs). All the junctions were innervated by unequal amounts of LPN/SN boutons. The range of fractional occupancies by the two nerves was between 39%/61% (smallest difference) and 13%/87% (the biggest difference). Intracellular recording showed that, on average, larger inputs produced larger amplitude EPP responses, and smaller inputs produced proportionally smaller amplitude EPP responses.

Fractional Occupancy:Fractional Synaptic Effect

As at 0 and 7 days, at day 14 there were strong correlations between the fractional occupancies and fractional synaptic effects of the LPN and SN inputs (Pearson's $r = 0.98$; Fig 5.8a). Consistent with data at the other recovery times, here, the confidence limits (0.83 to 1.0) indicated the correlation was highly significant ($P < 0.001$). Here, variance analysis indicated that the mean difference between each LPN and SN input's contribution to total synaptic effect and the regression line was 4.4% ($\pm 1.1\%$). Only four inputs showed contributions to synaptic size and effect which were outside the mean difference. For example, at one polyneuronally innervated endplate, the LPN was the minor input (39% of total size) but produced the majority of fibre depolarisation

(49% of total); the SN was the larger of the two inputs (occupying 61% of the endplate) but contributed less to total synaptic effect than expected (51%), based on its size. The amount of variance in the structure: function relationship for fractional occupancy: synaptic effect changed significantly during the two week period of recovery from TTX ($P < 0.05$, one way ANOVA with Tukey post hoc tests; Fig 5.9a). There were no mean differences between LPN and SN input variance over time, but there were significant increases in the number of LPN and SN inputs with disproportionate structure: function relationships between days 0 and 7 ($P < 0.05$ for both data sets) and a significant decrease in the number between days 7 and 14 ($P < 0.05$ for both data sets). However, there were no significant differences in the number of LPN or SN inputs with disproportionate structure: function relationships between days 0 and 14 ($P > 0.05$ for both data sets).

E:O Index

When inputs were normalised for size, very weak correlations between fractional occupancy and synaptic effect were revealed (Person's r for LPN inputs = 0.1; Pearson's r for SN inputs = 0.4; Fig 5.8b). However, this correlation was not significantly different from zero (*LPN confidence limits*: -0.69 to 0.81, $P > 0.5$; *SN confidence limits*: -0.52 to 0.90, $P > 0.05$). At day 14, the LPN and SN e:o indices showed little scatter from the regression line. The mean difference between each LPN e:o index and the regression line was 0.15 ± 0.03 ; the mean difference between each SN e:o index and the regression line was 0.06 ± 0.02 . There was a significant change, during recovery, in the number

of LPN and SN inputs expressing normal synaptic strength ($P < 0.05$ for LPN and SN inputs; one way ANOVA with Tukey post hoc tests; Fig 5.9b): between days 0 and 7, there were significant increases in the number of LPN and SN inputs with disproportionately strong or weak e:o indices ($P < 0.05$), which decreased significantly by day 14 ($P < 0.05$). There were no significant differences in the number of LPN or SN inputs with normal synaptic strength between days 0 and 14 ($P > 0.05$). However, at day 14, SN inputs did have, on average, significantly lower e:o indices than LPN inputs ($P < 0.05$).

Fractional Occupancy:Fractional Quantal Content

There were very strong, significant correlations between the fractional occupancy and quantal content of the LPN and SN inputs (Pearson's $r = 0.93$; *confidence limits*: 0.58 to 1.0; $P < 0.01$; Fig 5.8c). The variance analysis indicated that there was a reduction (from day 7) in the number of LPN and SN data points which fell away from the regression line: the mean difference between each input's contribution to total junctional quantal content and the regression line was 7.2% ($\pm 2.3\%$). However, further analysis showed that there were no significant differences in the fractional occupancy:quantal content relationship during recovery from paralysis ($P > 0.05$; parametric one way ANOVA, no post hoc tests performed; Fig 5.9c).

QC:O Index

Finally, when inputs were normalised for size, there were no significant correlations between input size and the amount of transmitter released at LPN and SN inputs (Pearson's r for LPN inputs=0.19, confidence limits: -0.65 to 0.83, $P>0.05$; Pearson's r for SN inputs=0.17, confidence limits:-0.67 to 0.82, $P>0.05$; Fig 5.8d). There was very little scatter in the data; the mean difference between each LPN input's qc:o index and the regression line was $0.23 (\pm 0.07)$ and the mean difference between the SN inputs and the regression line was 0.11 ± 0.04 . As for fractional occupancy:quantal content, there were no significant changes in the number of inputs showing normal synaptic strength during the two week recovery period (parametric one-way ANOVA; no post hoc tests performed; Fig 5.9d). However, the LPN inputs released significantly less transmitter than SN inputs at 0 days recovery from paralysis ($P<0.05$); by 7 days this difference was gone, and remained absent at day 14.

Fig 5.1 Morphological changes at polyneuronally innervated 4DL endplates during recovery from paralysis. Each parameter is given for 0 (n=6 muscles), 7 (n=8 muscles) and 14 (n=7 muscles) days after the resumption of activity. Error bars are \pm s.e.m. One-way ANOVAs and Dunn's post hoc tests were performed at the 5% significance levels within groups. * indicates a significant difference from 0 days recovery; ** indicate a significant difference from 7 days recovery. (a): There were no significant changes the mean size of endplates during recovery. (b): Terminal boutons grew throughout the recovery period, but the growth was only significant at day 14. (c): Fewer terminal boutons innervated each endplate as recovery progressed, but the reduction was only significant at day 14.

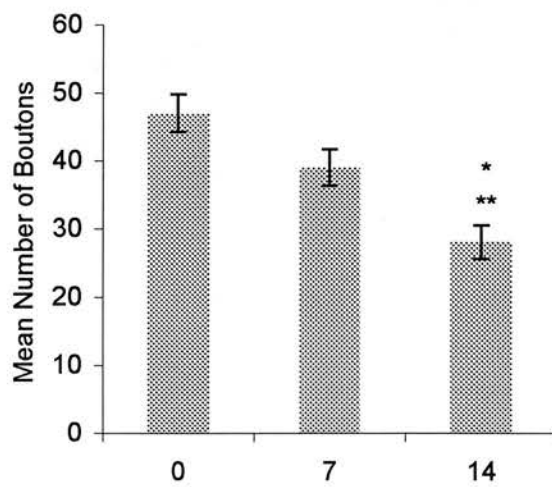
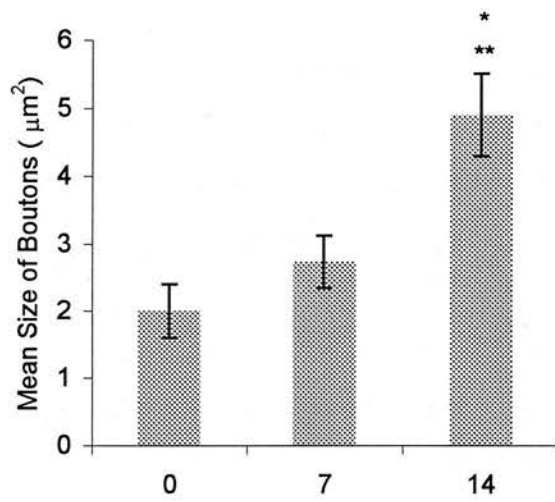
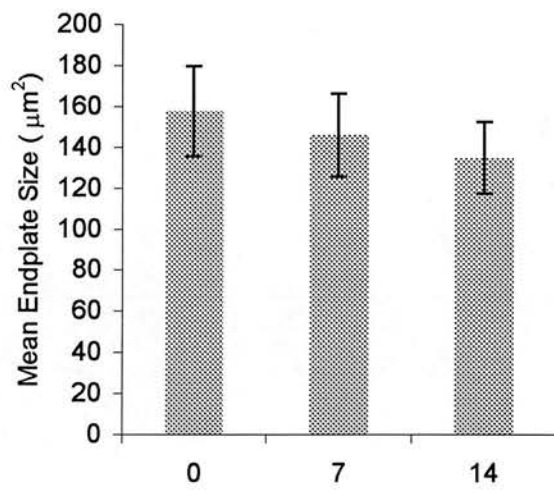


Fig 5.2 Qualitative changes in polyneuronally innervated 4DL endplates during recovery from paralysis. LPN and SN boutons were labelled with FM1-43 and RH414 at 0 (a), 7 (b) and 14 (c) days after the resumption of activity. There was no overall change in the size of endplates (in the panels in (a), 2 adjacent endplates are shown in each panel), but the number of boutons contacting each endplate declined with increasing recovery from paralysis and the boutons which were retained grew larger. At day 0, there were many more 50/50 polyneuronally innervated endplates. At day 7, note that in each example, the boutons belonging to the SN (left) or the LPN (right) appear more mature; the boutons belonging to the other nerve, in each case, are smaller and more numerous. By day 14, the innervation resembles mature, persistent polyneuronally innervated endplates (see **Fig 4.6**, for comparison). Scale bar is 20 μ m.

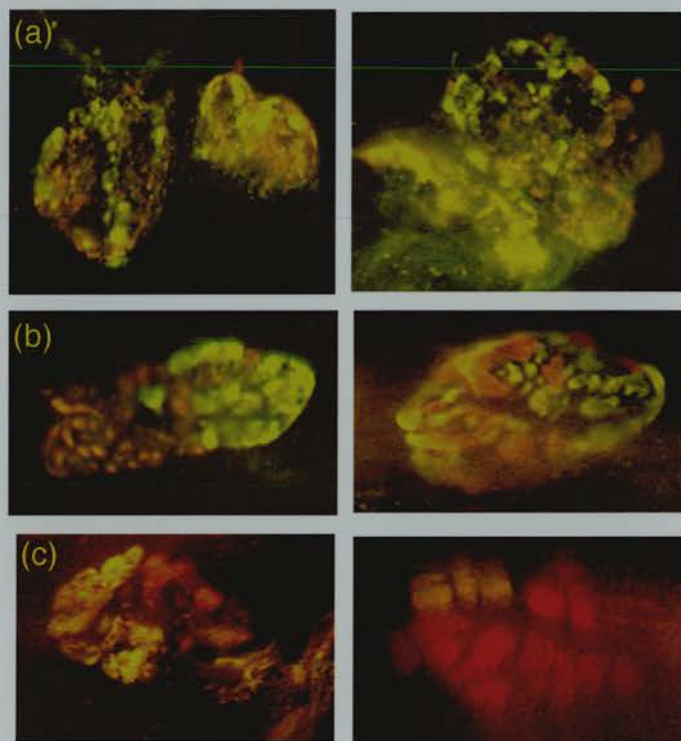
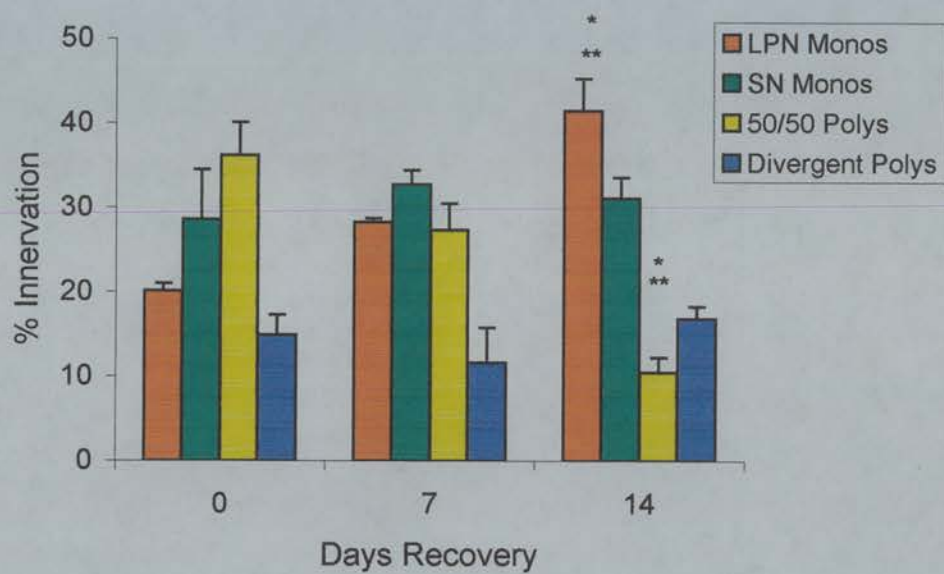


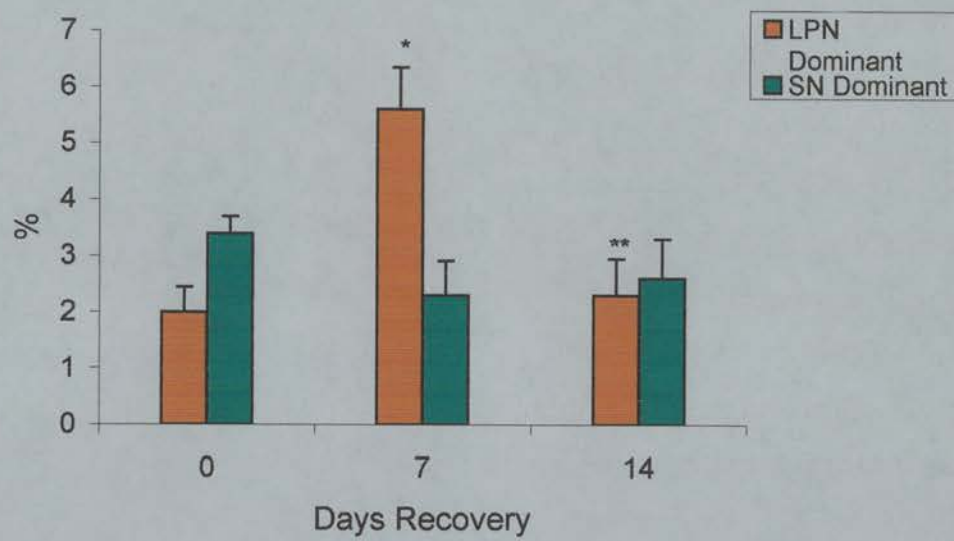
Fig 5.3 Alterations in 4DL innervation patterns during recovery from paralysis. At 0 (N=4 muscles; n=879 fibres), 7 (N=4 muscles; n=806 fibres), and 14 (N=4 muscles; n=813 fibres) days after activity resumed, the mean proportion of the endplates expressing each pattern of innervation is shown. Error bars are \pm s.e.m. One-way ANOVAs with Dunn's post hoc tests at the 5% significance levels were performed between groups. * indicates a significant difference from day 0, between groups; ** indicate a significant difference from day 7, between groups. There were no significant changes in the incidence of SN mononeuronally innervated endplates or the divergent polys. However, the proportion of LPN mononeuronally innervated endplates increased significantly by day 14 and the proportion of 50/50 polys decreased significantly over the same time course.



	0 Days	7 Days	14 Days
Innervation Pattern			
LPN Mononeuronal			
Mean (% fibres)	20	28.3	41.6
SEM (% fibres)	0.9	0.5	3.8
Comparison / P		0 vs. 7 / >0.05 (ns)	0 vs. 14 / < 0.001 7 vs. 14 / < 0.01
SN Mononeuronal			
Mean (% fibres)	28.1	32.9	31
SEM (% fibres)	6.0	1.7	2.5
Comparison / P		0 vs. 7 / >0.05 (ns)	0 vs. 14 / >0.05 (ns) 7 vs. 14 / >0.05 (ns)
50/50 Polyneuronal			
Mean (% fibres)	36.6	27.3	10.7
SEM (% fibres)	3.9	3.2	1.8
Comparison / P		0 vs. 7 / >0.05 (ns)	0 vs. 14 / <0.001 7 vs. 14 / <0.01
Other Polyneuronal			
Mean (% fibres)	15.2	11.5	16.7
SEM (% fibres)	2.4	4.2	1.5
Comparison / P		0 vs. 7 / >0.05 (ns)	0 vs. 14 / >0.05 (ns) 7 vs. 14 / >0.05 (ns)

Table 5.1. Innervation patterns in 4DL muscles change in the first two weeks of recovery from TTX activity block. Four muscles were taken at each time point and analysed for patterns of innervation. Patterns were divided into four groups: LPN mononeuronally innervated, SN mononeuronally innervated, 50/50 (LPN/SN) polyneuronally innervated, and all other distributions of polyneuronal innervation. Means and SEMs of the % of fibres in 4 muscles expressing the given pattern of innervation at each time point are presented. Changes in the number of fibres expressing the innervation pattern at each day were analysed by one way ANOVA with Dunn's post hoc tests. P values for 95% confidence intervals are given; non-significant differences are designated as (ns).

Fig 5.4 Alterations in the innervation patterns of divergent polys during recovery from paralysis. The group of divergent polys was subdivided into 2 groups: one in which the LPN supplied the majority of the endplate (orange bars) and one in which the SN supplied the majority (green bars). At 0 (N=4 muscles; n=48 fibres), 7 (N=4 muscles; n=63 fibres) and 14 days (N=4 muscles; n=39 fibres) after the resumption of activity, the mean proportion of the endplates expressing each pattern of innervation are shown. Error bars are \pm s.e.m. * indicates a significant difference from day 0, between groups; ** indicates a significant difference from day 7, between groups. There were no significant changes in the proportion of SN-dominant polys over time. However, the incidence of LPN-dominant polys increased significantly between day 0 and 7, and decreased significantly by day 14. There was no significant difference in the proportion of LPN-dominant polys found between day 0 vs. day 14.



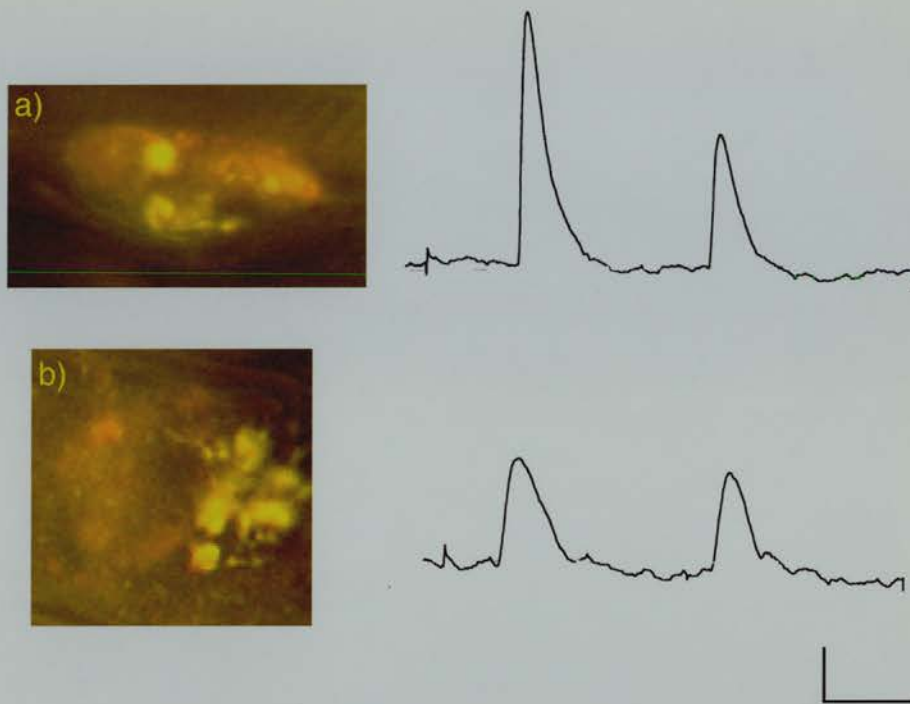


Fig 5.5 Morphology and electrophysiology of 4DL polyneuronally innervated endplates with no recovery from activity block. Left hand panels show LPN and SN inputs, labelled with FM1-43 and RH414. Right hand traces are examples of intracellularly recorded EPPs (from the LPN and SN, respectively) evoked after bathing nerve-muscle preparations in μ CTX to abolish muscle action potentials. a): The majority of the endplate was innervated by the LPN; the LPN also produced the larger of the two EPPs in response to nerve stimulation. The SN input to this endplate was proportionately smaller than the LPN, in terms of the size of the observed EPP. b): Here, both nerves supplied approximately equivalent areas of the endplate, and the amplitude of the EPPs were also approximately the same amplitude. Scale bars: 10msec/5mV (x and y axis, respectively).

Fig 5.6 Structure: function relationships at polyneuronally innervated 4DL endplates with no recovery from TTX paralysis. Convergent LPN (●; solid regression lines) and SN (▲; dotted regression lines) inputs were plotted in terms of their % contribution to (a) total junctional effect and size and (c) total junctional quantal content and size. Occupancy (% of total) was calculated as:

$$[\text{size of input}] / [\text{size of LPN input} + \text{size of SN input}] * 100$$

where inputs were measured in μm^2 . Synaptic effect (% of total) was calculated as:

$$[\text{EPP amplitude of input}] / [\text{LPN EPP amplitude} + \text{SN EPP amplitude}] * 100$$

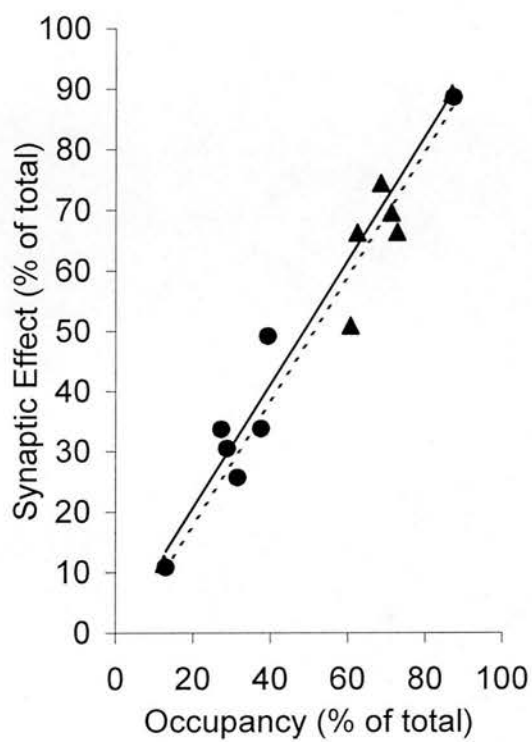
where EPP amplitude was measured as the mean of 100 responses to nerve stimulation. Quantal content (% of total) was calculated as:

$$[\text{QC of input}] / [\text{LPN QC} + \text{SN QC}] * 100$$

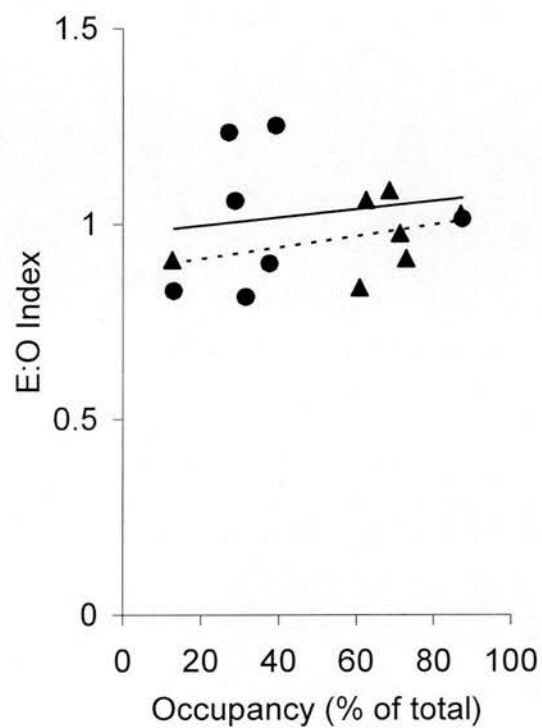
where QC was measured by the variance method (see section 2.4). In graphs (b) and (d), the structure: function relationships shown in graphs (a) and (c) were plotted after inputs had been normalised for size (see section 4.3.3 and 4.4.2 for the derivation of the equations used for the e:o and qc:o indices).

(a): Small LPN and SN inputs were significantly weaker than large inputs. (b): Small inputs were also significantly, disproportionately weaker than large inputs. (c): Small and large inputs were equally likely to provide the majority as the minority of total junctional quantal content: the observed relationships between input size and contribution to total junctional quantal content were not significant. (d): When LPN and SN inputs were normalised for size, no significant relationships between input size and amount of neurotransmitter released were revealed; small and large inputs showed comparable quantal contents.

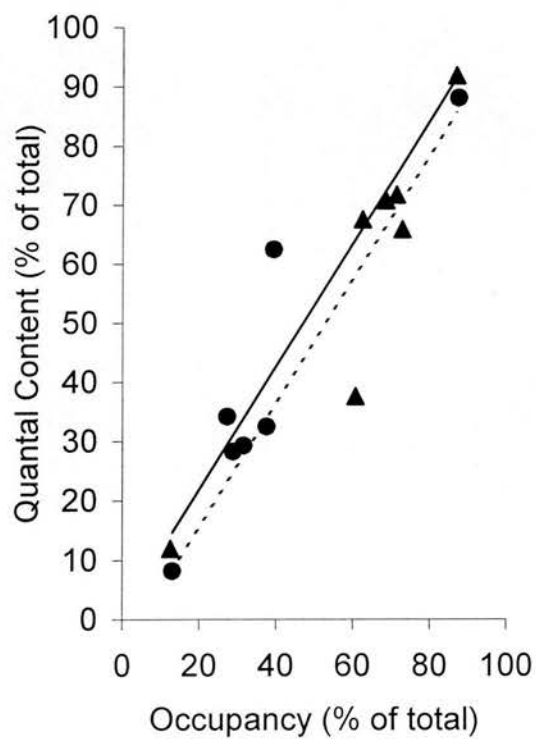
a)



b)



c)



d)

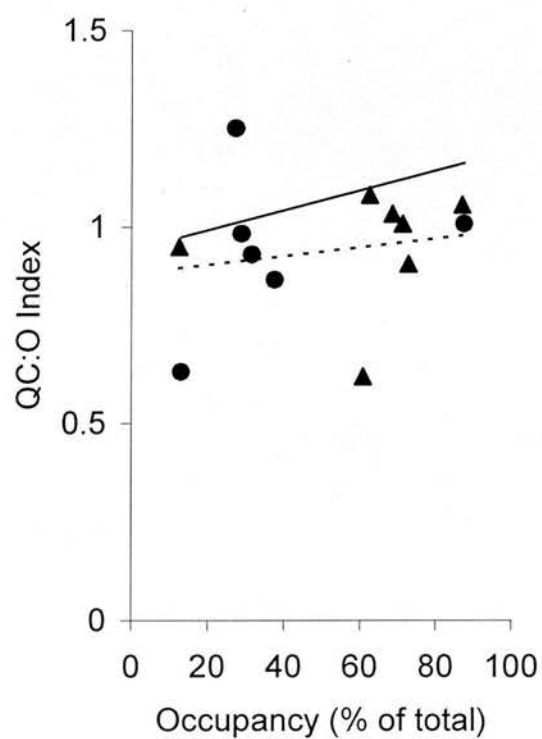


Fig 5.7 Structure: function relationships at polyneuronally innervated 4DL endplates 7 days after recovery from paralysis. LPN (•; solid regression lines) and SN (▲; dashed regression lines) inputs were plotted as % contributions to (a), total junctional size and effect and (c), total junctional size and quantal content, together with (b) the e:o indices and (d) the qc:o indices of the inputs (for a complete description, see the figure legend for **5.6**).

(a): Small LPN and SN inputs were weaker than large inputs, but the relationship was not as strong as for that at 0 days recovery. In addition, the correlations were not quite significant. (b): When the synaptic effects of LPN and SN inputs were normalised for size, there was no significant difference between the synaptic effect of small vs. large SN inputs, however, small LPN inputs were significantly stronger than large LPN inputs. (c): As at day 0, small inputs released less transmitter than large inputs. The regression line is presented as a single, heavy line to indicate the superimposition of the regression lines from both data sets. (d): In contrast to day 0, small inputs did not release disproportionately more (or less) transmitter than large inputs. In all cases, note that there is more scatter in the data around the regression line compared to the graphs presented **Fig 5.6**.

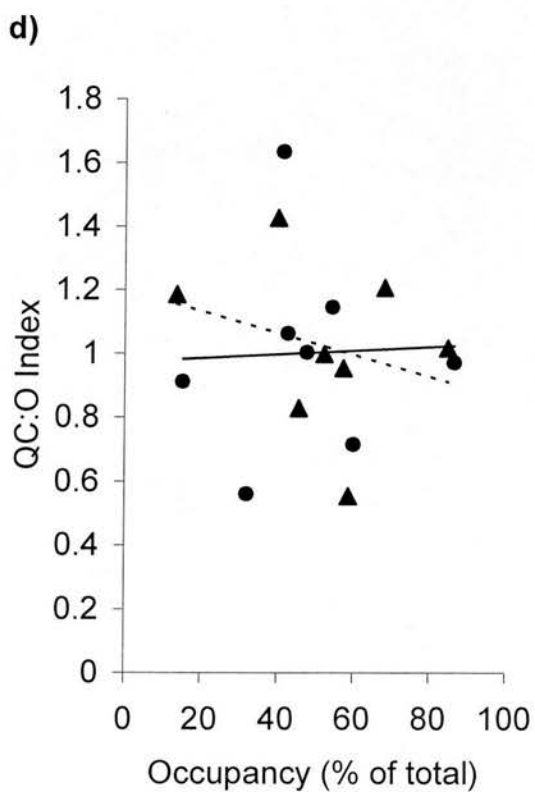
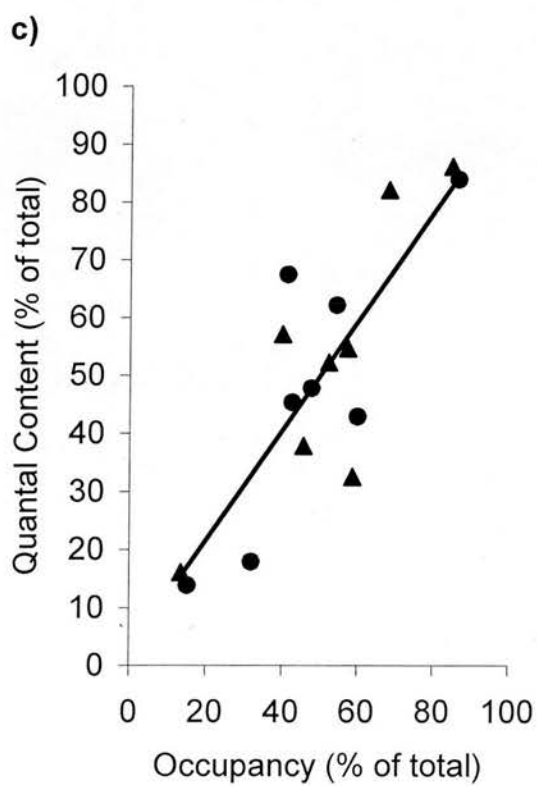
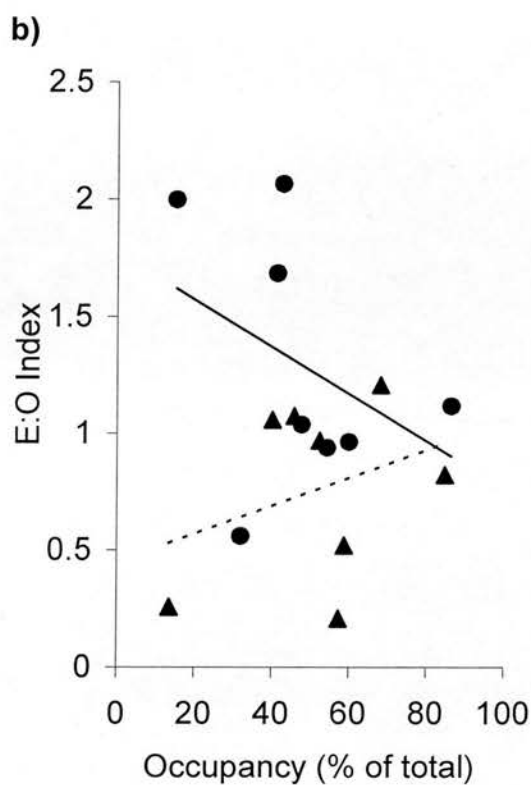
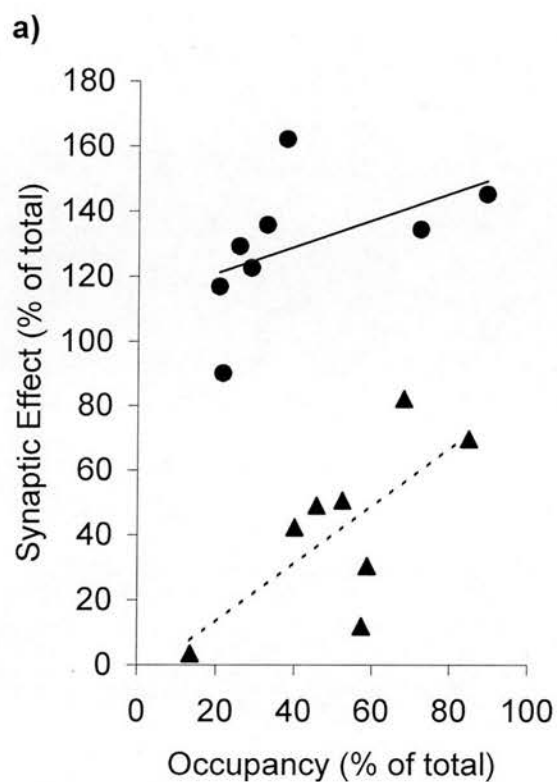
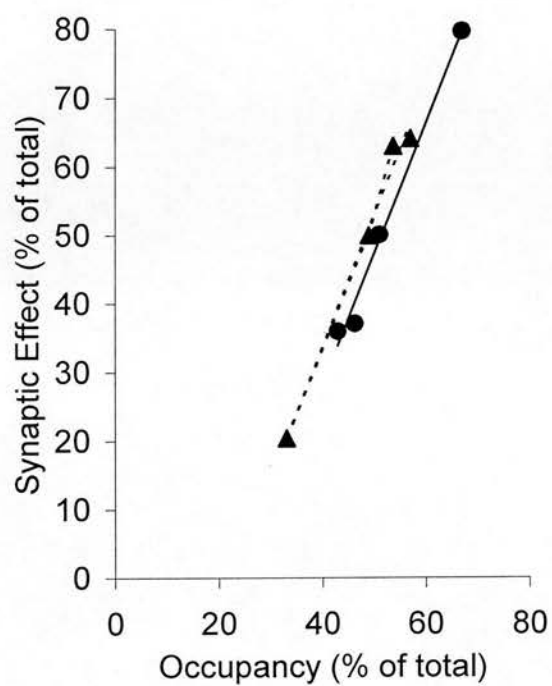


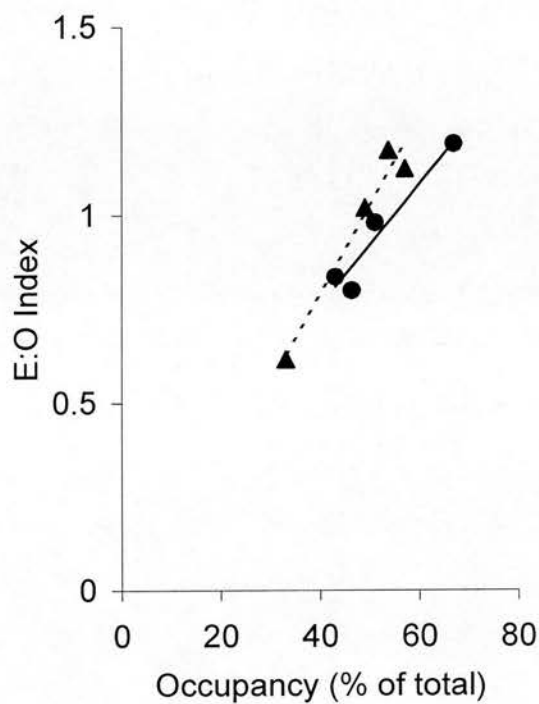
Fig 5.8 Structure: function relationships at polyneuronally innervated 4DL endplates 14 days after recovery from paralysis. As for the other recovery times, each LPN (•; solid regression lines) and SN (▲; dashed regression lines) input was plotted as % contribution to (a), total junctional size and effect and (c), total junctional size and quantal content, along with (b) the e:o indices and (d) the qc:o indices of the inputs.

(a): Like 0 and 7 days recovery, small LPN and SN inputs produced less of a synaptic effect than large inputs, 14 days after activity resumed. (b): When inputs were normalised for size, the e:o indices for small and large LPN and SN inputs were equivalent. (c): As at day 0 and 7, at day 14, small inputs released less transmitter than large inputs, but (d): small LPN and SN inputs did not release disproportionately less transmitter than large inputs, when relative size was taken into account. Again, in all cases, note the difference in the scatter of the data around the regression lines compared to the graphs presented Figs 5.6 & 5.7.

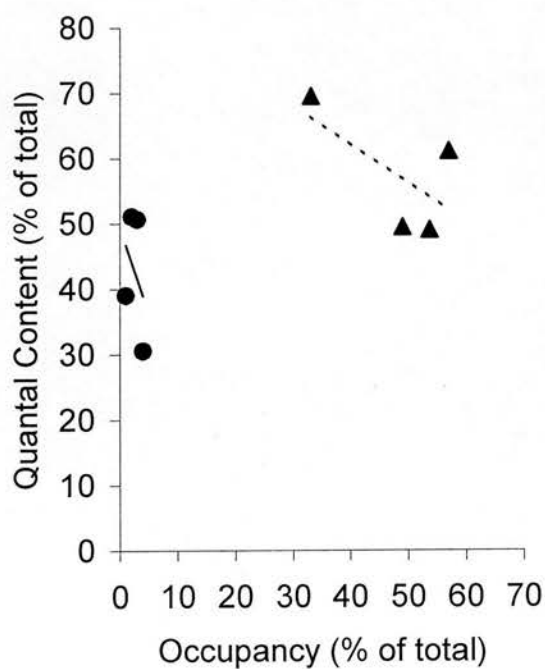
a)



b)



c)



d)

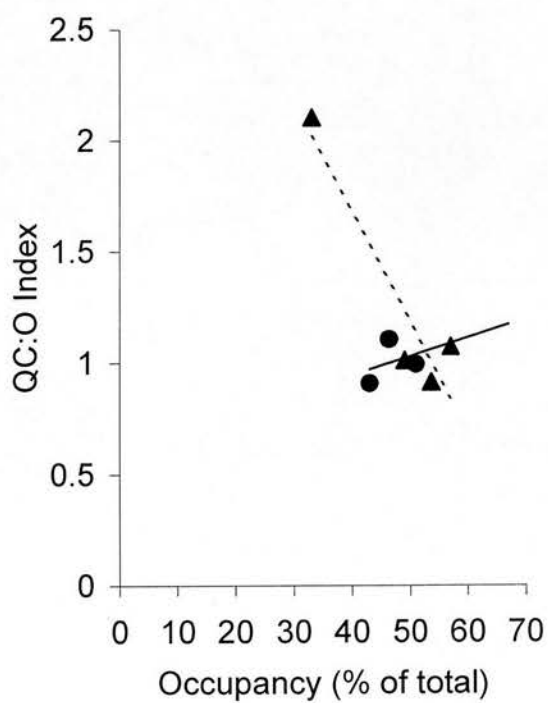
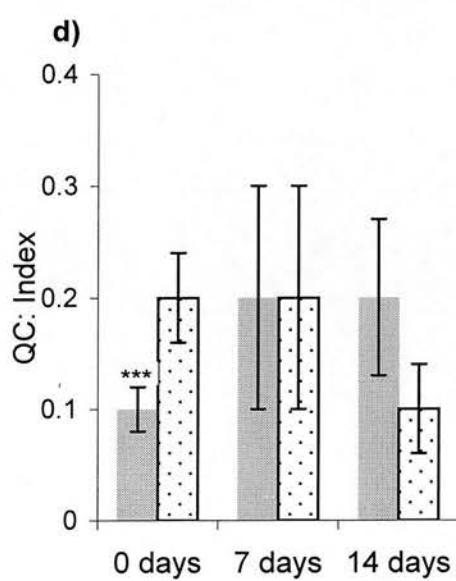
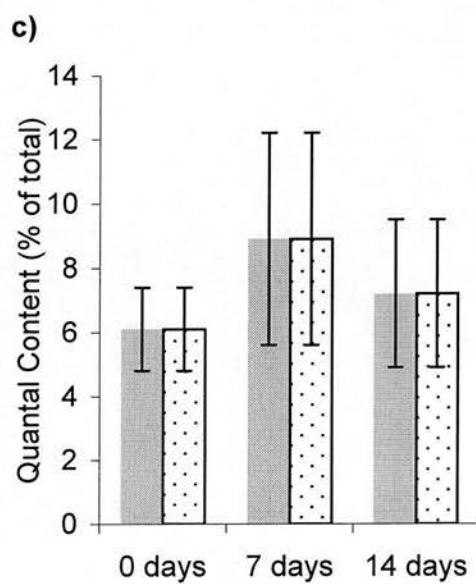
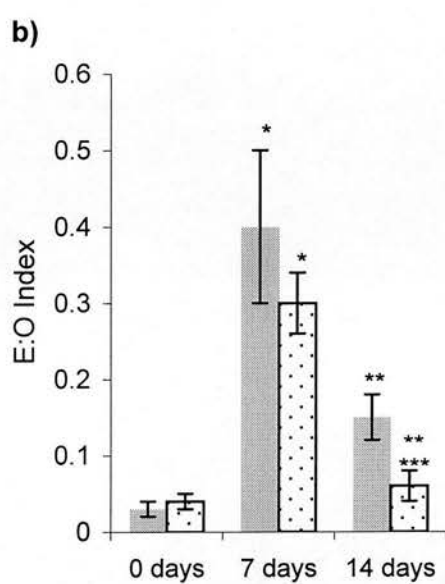
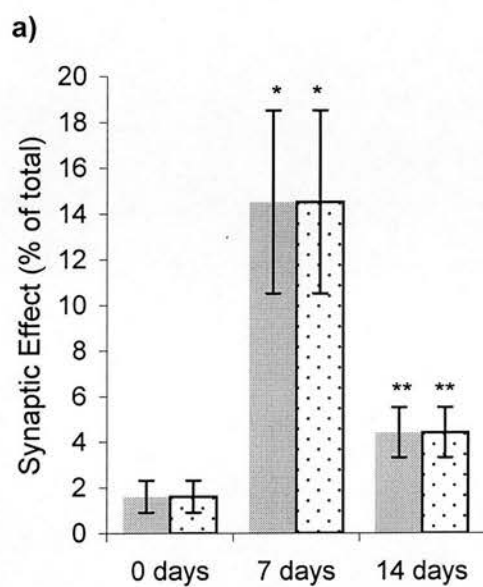


Fig 5.9 Variation in the number of convergent inputs with normal structure: function relationships during recovery from TTX paralysis. Each graph expresses the amount of scatter in the data from the regression lines in the graphs shown in Figs 5.6-5.8; each bar is the mean distance from the regression line for each group of inputs. Error bars are \pm s.e.m. The four parameters of synaptic strength (% total effect, e:o index, % total quantal content, and qc:o index; a-d, respectively) are shown; grey bars reflect LPN input data and spotted bars are data from SN inputs. One-way ANOVAs with Dunn's post hoc tests at the 5% significance levels were performed for within group differences. The following within group differences were tested for significance for each of the four parameters: LPN inputs at days 0, 7 and 14; SN inputs are days 0, 7 and 14, and LPN vs. SN inputs at days 0, 7 and 14. * indicates a significant difference from 0 days; ** indicates a significant difference from 7 days; *** indicates a significant difference between LPN and SN inputs.

a): There was a significant increase in the number of inputs where fractional occupancy was *not* in proportion to synaptic effect between days 0 and 7 (in other words, there was a decrease in the number of inputs with proportional size and effect). By day 14, the number of inputs with disproportionate contributions to total junctional effect had declined significantly, and was not significantly different from day 0. b): Likewise, when input size was normalised, there was a significant increase in the number of inputs where the e:o indices were disproportionately strong or weak, between 0 and 7 days recovery. There were significantly more inputs with normal synaptic strength at day 14 than at day 7. The number of inputs with normal e:o indices were not significantly different between days 0 and 7. c): There was little variation in the number of inputs where fractional occupancy was in proportion to fractional quantal content. Statistical analysis showed there were no significant differences between days 0, 7 and 14. d): Likewise, when inputs were normalised for size, there were no significant changes in qc:o indices during the recovery period.



Discussion

In the experiments described in this chapter, the structural and functional changes that accompany synapse elimination in reinnervated 4DL muscles were examined. There are two points of general interest established by the results. First, it appears that much of the synapse elimination that occurs during recovery from paralysis is the result of LPN axons successfully gaining exclusive innervation of (formerly) polynuronally innervated endplates. Second, the results show that at early to mid-recovery times, there was some variability in the number of inputs with normal structure: function relationships. By the end of the period of recovery – as with the results described in **Chapter 4** – the majority of inputs displayed synaptic strengths that were in proportion to their sizes. These observations suggest that inputs which can achieve normal synaptic strength are preserved in 4DL muscles. Further, since a minority of inputs showed synaptic strengths that were not in proportion to their sizes, a critical period for synapse elimination may also operate after reinnervation and recovery from paralysis, beyond which all inputs are stable in their patterns of innervation.

5.7 Alterations in the Growth of Boutons

There were striking changes in the morphology of nerve terminal boutons during recovery from paralysis, with no concomitant changes in endplate size (see **Figs 5.1 & 5.2**). There are two possible explanations for the observed growth of boutons, with the concurrent decline in the number of boutons per endplate. The simplest account is that some inputs were able to elaborate the sizes of their nerve terminal boutons, at the expense of other inputs, which were removed from the endplates. This explanation agrees with other studies that show that during reinnervation,

regenerating axon terminals most often grow at the expense of sprouted terminals, some of which are eventually withdrawn from the shared endplates (Bennett & Raftos, 1977; Brown & Ironton, 1978; Slack & Hopkins, 1982). The second alternative is that the small boutons coalesced over time to produce fewer, but larger boutons. A related consideration is that the appearance of many small boutons at early to mid-recovery times actually reflected a constant number of boutons with changes in the clustering of the synaptic vesicles contained within. Because the styryl dyes label recycling vesicles, they reveal the location of clusters of vesicles, and not necessarily the location of discrete nerve terminal boutons. Although the stepwise changes that accompany synaptogenesis at reinnervated junctions have not been fully described, a body of work has detailed the changes in synaptic vesicle clustering that occur during development. Nerve terminals at newly formed synapses bear few synaptic vesicles and no ultrastructurally recognisable active zones. Subsequently, active zones appear, synaptic vesicles increase in number and become clustered at the active zones, and the nerve terminal becomes polarised (Kelly & Zacks, 1969; Takahashi et al., 1987; Buchanan et al., 1989). However, the clustering of the vesicles (that is, the distribution of vesicles within the boutons) does not seem to change during this time. Thus, although it is not possible to determine which of the above alternative explanations is correct based on the data presented here, a study using electron microscopy to identify the numbers of boutons and patterns of synaptic vesicle clustering could resolve this question.

5.8 Alterations in Innervation Patterns

The most obvious change in innervation patterns during recovery from paralysis was the loss of polyneuronal innervation, assessed by morphological criteria. The fall from 52% ($\pm 6.3\%$) to $27.4\% \pm (3.2\%)$ agrees with observations made in the 4DL by Barry & Ribchester (1995), who used intracellular recording and tension measurements to determine levels of polyneuronal innervation.

There were also striking changes in the patterns of innervation during the two week period (see Fig 5.3): there were no changes over time in the levels of either the SN mononeuronally innervated endplates or divergent polys, but there was an increase in the proportion of LPN mononeuronally innervated endplates and a decrease in the levels of 50/50 polys. The fact that there were no significant changes in any innervation patterns between days 0 and 7 suggests that if synapse elimination began in the first week of recovery, it did not accelerate until the second week of recovery. This time course is not particularly surprising: within a single muscle, the time at which individual fibres lose inputs is spread out over many days. For example, in the rat diaphragm, approximately 5-15% of the fibres become singly innervated on each postnatal day until all are singly innervated at two weeks of age (Redfern, 1970; Rosenthal & Taraskevich, 1977). In addition, junctions with two equally efficacious inputs can persist for many days in mice, before the balance tips in favour of one input, and the process of skewing accelerates (Colman et al., 1997). Likewise, in frogs, multiple innervation may persist for some time, with evenly matched inputs, before remodelling is triggered (Werle & Herrera, 1988; Herrera & Werle, 1990). Thus, synapse elimination in the 4DL is perhaps a protracted process, with subtle

modifications taking place at early times, before obvious changes are apparent in the muscles at later stages.

The simplest explanation for the shift in patterning is that the LPN was better able to compete and acquire endplate territory from the SN, while the SN was concerned with maintaining and consolidating the territory already established prior to the resumption of activity. The process may be explained competitions for innervation of the 50/50 polys were tipped in favour of the LPN, and the endplates progressed to being mononeuronally innervated (by LPN axons). This would be consistent with the observation that there were no changes over time in the levels of divergent polys. That is, the rate of endplates being transformed from a 50/50 to a divergent poly pattern was the same as the rate of endplates being transformed from a divergent poly to an LPN mononeuronal pattern of innervation.

It was inferred that the SN was less successful than the LPN in winning innervation of 50/50 poly endplates because, if the SN was equally likely to win these competitions, an increase in the levels of divergent polys and/or mononeuronally innervated SN endplates should have been apparent by day 14 of recovery. There are several possible explanations for the SN being less capable than the LPN at expanding its synaptic territory within the muscles after the return of activity. A discussion of the possibility that regenerating axons have a competitive advantage over intact inputs through their preferential access to neurotrophic factors was presented in a previous discussion (see section 3.16). Alternatively, it may be that once the SN has sprouted maximally, metabolic constraints prevent the axons from

further successful elimination of LPN inputs. Previous studies show that following partial denervation by LPN crush, the SN sprouts maximally to innervate the 4DL (Ribchester, 1988; Barry & Ribchester, 1995). Similar results – with respect to the time course of sprouting – were found in the preceding chapter (see section 4.6). However, Thompson & Jansen (1977) showed that there is an upper limit to the capacity for nerve terminal sprouting in the rat soleus muscle. Brown & Ironton (1976) noted a similar limit to the capacity for sprouting in denervated mouse peroneus tertius muscles. It appears that motor nerve terminals will seek out and innervate denervated muscle fibres as long as the axons do not exceed the maximum number of endplates that they can maintain. Metabolic constraints have also been proposed to account for the non-competitive synapse elimination that occurs during development. There have been reports that a reduction in the size of motor units takes place in muscles that have been partially denervated soon after birth, even though competition for innervation is reduced in the muscles, and a consequence of the synapse elimination is that some fibres are left completely denervated (Brown et al., 1976; Thompson & Jansen, 1977). Such a mechanism presumes that terminal arbours impose a metabolic load on motor neurons that in turn affects the neuron's ability to supply its synapses with the materials needed for growth. When the metabolic load is less than an optimal baseline level for the neuron, all the terminals may grow and compete effectively for endplate innervation. When the load exceeds the baseline level, the terminals will tend to be placed at a competitive disadvantage and retract. Consistent with this idea, computer models have been generated which simulate mechanisms of synapse elimination and synaptic competition at developing neuromuscular junctions (Willshaw, 1981; Van Essen et al., 1990). These have

showed that a metabolic load mechanism can achieve synapse elimination without the wholesale denervation of endplates. It is an attractive idea that the potential number of synaptic terminals that a motor neuron could maintain would exceed the actual or expected number of synapses made with the target muscle, with a substantial degree of safety.

Finally, in the group of divergent polys, the levels of LPN dominant polys shifted during recovery: increasing at day 7, and decreasing back to levels comparable to day 0, when examined on day 14. This provides further support for the suggestion that the LPN won more competitions for innervation of the endplates than the SN, given above. To extend this description, it may have been that between days 0 and 7, the LPN was most successful at tipping the balance of innervation at the 50/50 poly endplates in its favour. Because there were no significant changes in innervation patterns until day 14, it follows that between days 7 and 14, the rate of transformation from LPN dominant polys to mononeuronally innervated LPN endplates exceeded, but did not replace the rate of tipping. Colman & Lichtman (1997) have estimated the time required for endplates to become singly innervated once the synaptic efficacies of the convergent inputs has diverged. They concluded that once the quantal contents of the inputs had been separated by a factor of 4, single innervation would result within 24 hours. The rapid nature of these changes may explain the significant increase at day 7 and subsequent decline by day 14 of LPN dominant polys (in the divergent poly group). It is noteworthy that there were no changes in the proportion of divergent polys that were mostly innervated by the SN (see Fig 5.3). This, together with the lack of change in the levels of mononeuronally

innervated SN endplates during recovery, suggests that the SN was effective in maintaining its distribution of innervation in the muscles. Thus, it may be that the competition for endplate occupancy and observed synapse elimination occurred at the 50/50 polys, and was largely, if not entirely due to the LPN tipping the balance of synaptic efficacy:occupancy in its favour before transforming the endplates to being innervated exclusively by LPN axons.

5.9 Alterations in Structure:Function Relationships

That there was an increase in (1) the number of inputs with proportional synaptic occupancy:efficacy relationships and (2) normal e:o indices as recovery progressed suggests that inputs without such relationships were being eliminated. Thus, it seems that an appropriate structure:function relationship may indeed protect small inputs from elimination. Further, the observation that there was no difference between the synaptic effect of inputs at 0 days and 14 days recovery supports activity as being the trigger for synapse elimination. It is noteworthy that there were no significant changes in the amount of transmitter released by convergent inputs during recovery, suggesting that synaptic preservation may be regulated at the level of the postsynaptic membrane.

These results provide new evidence on the nature of the competitive changes that occur during synapse elimination. Colman et al. (1997) noted a progressive skewing between the synaptic efficacies of convergent inputs during synapse elimination, and concluded that the inputs with the strongest efficacies were those which were maintained at mononeuronally innervated endplates. However, the authors did not

investigate the correlation between synaptic size and efficacy and so it was unclear from this study if inputs with smaller efficacies were proportionately or disproportionately smaller in size. The results in the preceding chapter indicated that most inputs showed proportionate e:o and qc:o indices after long recovery times. However, it was possible that small inputs were being eliminated while releasing neurotransmitter in proportion to their sizes. The present results support the idea that small inputs are able to "fight back" against large inputs by achieving normal structure:function relationships. Perhaps if one of the inputs to a polyneuronally innervated endplate starts to gain efficacy by growing, but not as much as the other input does by shrinking, the inputs can exist in a stalemate. Thus, it may be that activity perpetuates a continuous competitive relationship between inputs to polyneuronally innervated endplates; however, if the overall structure:function relationships are equivalent, activity alone is not sufficient to induce the elimination of either of the inputs.

5.10 A Putative Critical Period

The results also suggest that a normal structure:function relationship may not be the only factor which underlies the preservation of polyneuronal innervation. In both the preceding and current results, there were clearly inputs which showed synaptic strengths that were not in proportion to their sizes. Barry & Ribchester (1995) have suggested that persistent polyneuronal innervation in reinnervated muscle may be the result of some inputs surviving a critical period for synapse elimination. The existence of outlying e:o and qc:o indices in the present work may be explained by such a mechanism. It has been shown in a variety of structures and pathways that

early experience plays an important role in shaping the adult organisation of the nervous system. A well known example is the effect of early visual experience on the formation of the ocular dominance column in the primary visual cortex (Wiesel & Hubel, 1965; Hubel & Wiesel, 1970). Occluding one eye in kittens for a short period after birth permanently disrupted the normal organisation of ocular dominance columns, despite months of normal visual experience between eye occlusion and testing. Importantly, monocular deprivation in adulthood did not similarly alter the organisation of inputs to the visual cortex. In short, Hubel & Wiesel defined an early critical period during which visual experience can determine how the adult visual cortex is organised. This period coincides with the period of synapse elimination in which originally overlapping inputs from each eye segregate to form the ocular dominance columns (Levy, Stryker & Shatz, 1978). Early monocular deprivation presumably disrupts the development of the ocular dominance columns by altering the normal course of synapse elimination. The end of the critical period for monocular deprivation is determined, apparently, by the end of synapse elimination.

There have been few reports of the existence of critical periods for synapse elimination at the neuromuscular junction. Androgen has been shown to act during early critical periods to prevent synapse elimination in the levator ani muscle (Jordan et al., 1990; Jordan et al., 1989a & b; Lubischer et al., 1992) and developmental synapse elimination can be delayed by inactivity (Thompson et al., 1979; Brown et al., 1981, 1982; Caldwell & Ridge, 1983). This delay after a period of inactivity differs from the present results, as well as from the effects of androgen, because the former effects were transient, whereas the polyneuronal innervation described here

(and that described under androgen treatment) appears permanent. On the other hand, the results are common, in that each experimental intervention must be given before the end of synapse elimination in order to influence polyneuronal innervation in skeletal muscle. A common critical period, during which factors such as androgen and activity can modify synapse elimination, suggests that the mechanism(s) underlying neuromuscular synapse elimination may normally only be transiently active during perinatal life and after reinnervation.

5.11 Proteases May Exert Effects During a Critical Period

Although these mechanisms are as yet unknown, a role for proteases in synapse elimination has been proposed by several authors (O'Brien et al., 1978; Vrbová et al., 1988). Liu et al. (1994a & b) have suggested that the muscle fibre releases proteases into the synaptic cleft whenever the muscle fibre is activated. The action of proteases may be to destabilise the junction between nerve terminal boutons, the synaptic basal lamina, and terminal Schwann cells, which would ultimately lead to the retraction of inputs which had been subjected to the highest levels. On the other hand, presynaptic activity was proposed to cause the release of protease inhibitors, which protect the more active input. Thus, local modulation of proteolytic activity may influence the outcome of activity-dependent synaptic competition by inducing the loss of inputs that are less active than their competitors. One limitation of such a mechanism is that the outcome of competition would seem to depend only on the overall activity level of an input as opposed to the relative activity among the competing inputs. In addition, it is difficult to imagine how proteolytic activity

might be locally modulated within a neuromuscular junction so that some boutons are lost, while adjacent ones, a few microns away, are protected.

Nonetheless, *In vivo* application of protease inhibitors has been shown to delay synapse elimination during development (Connold et al., 1986) and following partial denervation (Vrbová & Fisher, 1989). Recently, Zoubine et al. (1996) focused on the role of thrombin and thrombin-like molecules in synapse elimination. After delivering the thrombin-specific inhibitor, hirudin, for several days during early postnatal life, Zoubine et al. observed that multiple innervation was retained for several days. Since hirudin – isolated from the leech – is not present in mammalian tissues, it remains to be determined whether endogenous protease inhibitors have similar effects *in vivo* in mammals. It is also uncertain whether thrombin's proteolytic activity influenced synapse elimination directly, or just delayed the transition from multiple to single innervation. However, the paper by Zoubine et al. raises the question that proteases may be one of a number of factors released from muscle fibres during a critical period of synapse elimination.

5.12 Future Work

The above discussion about proteases raises interesting possibilities for extending the present work and the paper by Zoubine et al. (1996) provides a starting point. For example, the ways thrombin and related molecules lead to synapse loss could be investigated using the present experimental paradigm. Prothrombin mRNA is widely expressed throughout the adult nervous system (Dihanich et al., 1991), but whether or not prothrombin is expressed in motor neurons, skeletal muscle fibres or in other

cells present at the neuromuscular junction is unknown. Activation of thrombin receptors by thrombin has been shown to induce retraction in neurons (Suidan et al., 1992) *in vitro*. Using the present experimental design, it should be possible to focally apply thrombin/hirudin to nerve terminal boutons *in vitro* at varying stages of recovery from paralysis. This may resolve questions about whether boutons of a particular size are (more) adversely affected by proteases at different stages of synapse elimination. In addition, such an investigation may reveal the site of action of proteases during synapse elimination: these molecules may primarily influence the disassembly of presynaptic terminals, or perhaps also the disassembly of postsynaptic specialisations.

Finally, the temporal changes that occur when inputs are achieving normal synaptic strength may be of interest for further investigation. Recently, Zhang et al. (1998) demonstrated that in the developing visual system of frogs, the repetitive correlated spiking of neurons resulted in potentiation or depression of transmission, depending on the temporal order of the activity among various synaptic inputs. Their results showed that a subthreshold input became strengthened when it was activated within 20msec of a suprathreshold input; if the subthreshold input was activated up to 20msecs *after* the suprathreshold input, it became further depressed. This result may have implications for understanding how convergent LPN/SN inputs achieved normal synaptic strength in the present experiments. It should be straightforward to test such temporal relationships using the current paradigm during the range of recovery times *in vitro*.

Chapter 6: General Discussion

New Perspectives On the Role of Activity in Nervous System Plasticity

The function of the nervous system depends on the underlying detailed and highly stereotyped patterns of neural connections. How these precise patterns of synaptic connections form is not known, but activity dependent and independent processes clearly play a role. Throughout the nervous system, experiments have suggested that two broad mechanisms work in concert to orchestrate the formation of precise patterns of neural connections: those that require activity and those that do not. In general, it currently appears that the processes of pathway and target selection by growing axons take place via activity-independent mechanisms and that connections are subsequently refined by activity. However, recent evidence from several parts of the nervous system suggests that this might not always be the case.

The process of activity-dependent synaptic plasticity does not stop at birth, but continues throughout the lifetime of the organism as the patterns of neural activity driven by input from the external world continue to modify the strength and structure of dendrites, axonal arbours, and synapses (for examples, see Purves et al., 1986; Bailey & Chen, 1989). In contrast to prevalent ideas surrounding the primacy of activity as the refiner of synaptic connections in the adult, new results demonstrate that activity is not always required for synaptic modification and does not always produce it in predictable ways. This is epitomised by the results presented here, in which manipulating activity produced much greater or much less plasticity at 4DL neuromuscular junctions than was predicted by current assumptions about the mechanisms responsible for synapse elimination from skeletal muscle.

6.1 Different Pathways Have Different Activity Requirements for Appropriate Synaptogenesis

Early studies of neuromuscular development led to the currently accepted idea that the initial steps of pathway finding and target selection are activity-independent. This issue was first examined by Twitty and colleagues in the 1930s when they discovered that the embryos of a California newt, *Taricha torosa*, harboured a toxin (TTX; Mosher et al., 1964) that blocked neural activity in other amphibians without affecting their own sodium channels (Twitty, 1937). When pieces of *Taricha* embryos were grafted to the embryos of salamanders, the host embryos were completely paralysed for a period of days until about the stage that the larvae would normally begin to feed independently. At this time, the toxin wore off and the animals soon began to swim and feed in a remarkably normal fashion (Twitty & Johnson, 1934). Evidently, in the absence of neural activity, motor neurons had found and recognised their correct muscle targets, and appropriate pattern-generating circuits had developed within the spinal cord, because these animals, released from the toxin-induced paralysis, could swim and walk in a fashion that was indistinguishable from a normal salamander.

Similarly, in *nic-1* homozygous mutant zebrafish embryos in which the function of muscle AchRs is blocked (Westerfield et al., 1990), primary motor neurons establish normal patterns of innervation with their appropriate muscles, further suggesting that transmitter-mediated nerve signalling is not required for the development of neuromuscular specificity in this organism. Moreover, muscles are appropriately innervated (Liu & Westerfield, 1990), and synapses form (Dahm & Landmesser,

1991) when neural activity or neuromuscular transmission is blocked. Not only can motor neurons find their correct muscles in the absence of activity, but muscle spindle sensory neurons make specific connections with the appropriate motor neurons in the amphibian spinal cord in the absence of patterned neural activity (Frank, 1990).

More recently, studies from other parts of the nervous system have begun to provide exceptions to the rule that normal development occurs independently of activity. For example, the spatial pattern of connectivity found in the rodent somatosensory system can develop normally in the absence of activity, but the subsequent function is altered when activity is blocked during early development. The whiskers or vibrissae of rodents are innervated by separate vibrissal nerves, which are activated by movements of the whiskers. The arrangement of the whiskers on the face is reflected topographically in a distribution of discrete functional units, called barrels, in the cortex. Early experiments in which activity was blocked by chronic infusion of TTX to the vibrissal nerves or to the area of the cortex containing the barrels showed that inactivity did not prevent the emergence or arrangement of the barrels during development (reviewed by O'Leary et al., 1994). However, another study has produced contradictory results, suggesting that the method of manipulating activity levels during development may be important to the outcome. This raises the possibility that even before the onset of action potentials, other forms of activity may contribute to the development of synaptic connections. This study showed that in mice lacking the NR1 subunit of the NMDA receptor, barrels did not form during development (Li et al., 1994). However, these results may be difficult to interpret

because it is possible barrel formation is slowed in NR1 knockout mice; since the mice die shortly after birth, this possibility cannot be tested. Consistent with this possibility, pharmacological blockade of the NMDA receptor from birth did not prevent barrel *formation* (Henderson et al., 1992), but another recent paper indicates that NMDA activity is necessary for the appropriate *function* of barrels (Fox et al., 1996). In this study, when cortical cells were chronically blocked with glutamate receptor antagonists during development, the anatomical arrangement of barrels appeared unaffected. However, physiological analysis indicated that the function of the barrels was indeed abnormal. These results suggest that the effects of activity may be subtle, and that the two types of mechanisms – activity-dependent and independent – do not necessarily occur in two distinct and mutually exclusive time periods. Rather, these studies show that the two mechanisms may overlap and interact during development, each being responsible for different aspects of appropriate synaptic connectivity.

The strict activity-independence of development in the visual system has also recently come into question. During development, retinal axons use activity independent mechanisms initially to establish projections with the lateral geniculate nucleus (LGN) in the optic tectum: when activity is blocked, these projections form into a coarse-grained topographic map, but fail to be remodelled into a fine-grained map (reviewed in Constantine-Paton et al., 1990). However, the next step in development of the visual system has recently been shown to be activity dependent (Catalano & Shatz, 1998). During the wiring of connections between the LGN and cortex in mammals, there is an intermediate step in which thalamic axons grow and

interact with a population of neurons in the subplate before they contact their ultimate target neurons within the cortical plate. Catalano & Shatz (1998) blocked activity with TTX during this stage and subsequently found that the majority of axons projected to the (incorrect) subplate target, and not to the (correct) cortical targets. These studies highlight the extent to which different parts of the developing visual system may achieve their specific needs using common activity dependent and independent mechanisms, but employing them to different degrees and at different times. As discussed in Chapter 5, it may be the case that activity operates during critical periods of plasticity. Critical periods have also been shown to mediate activity dependent plasticity in the visual cortex (Hubel & Wiesel, 1965) and cerebral cortex (van Huizen et al., 1987).

6.2 Effective Synaptic Modifications Can Be Induced in the Absence of Activity in Several Parts of the Nervous System

Following the initial target selection and formation of synaptic contacts in the developing or regenerating nervous system, a refinement of connections commonly occurs. This usually takes the form of a reduction in the number of inputs that innervate the postsynaptic target (skeletal muscle: see section 1.5; autonomic ganglia: Lichtman, 1977, 1980; cerebellum: Crepel & Mariani, 1976; Crepel et al., 1976). A major purpose of this process may be to regulate the number and distribution of synapses made within that target. The initial redundancy and subsequent elimination of some innervation is a strategy that is probably well suited to ensuring both the rapid innervation of all the cells in a target and ultimately, a quantitatively appropriate distribution of synapses among the target cells. As has

been described in this dissertation, the process of synapse elimination is strongly influenced by activity. While this may be the general trend in the nervous system, the results presented in Chapter 3 suggest that not all circuits develop in the same way. These experiments show that synaptic modifications directed toward the establishment of the normal adult pattern of neuromuscular innervation can sometimes occur via activity-independent mechanisms. The results reveal a role for other factors in synapse elimination, which further suggests that it may be oversimplistic to draw a strict division between activity dependent and independent contributions to the final patterns of neural networks in the mature nervous system.

Advantageous functional changes at synapses have been suggested to underlie such complex phenomena as learning and memory. Perhaps the best described example of such changes is that of the long-term potentiation (LTP) of synapses in the hippocampus following a brief period of high frequency stimulation (Lømo, 1966). Since this early description of a long-lasting enhancement of transmission, LTP has been found in all excitatory pathways in the hippocampus (reviewed by Bliss & Collingridge, 1993), and in many other regions of the brain, including the visual cortex (Artola & Singer, 1987), motor cortex (Baranyi et al., 1991), and basal ganglia (Pennartz et al., 1993).

Classically, LTP has been induced by (activity dependent) stimulation regimes and/or pharmacological applications that result in strong depolarisations of the postsynaptic membrane. However, recently, there have been reports of LTP in

different regions of the brain which were induced independently of activity *per se*¹. For example, application of the retrograde messenger arachidonic acid by itself potentiated synaptic currents through AchRs in *Xenopus oocytes* and in hippocampal slices (Nishizaki et al., 1999). Non-activity dependent LTP has also recently been described at the mammalian superior colliculus (SC). The SC is a visual nucleus, which can be divided functionally into superficial and deep layers. It contains topographic maps which are susceptible to plasticity in the neonate and the adult (Withington et al., 1994). Platt et al. (1998) showed that the application of glycine to SC slices in the guinea pig produced a novel form of LTP that was neither mediated by nor dependent on depolarisation. Although the authors pointed out that long-lasting glycine release is not likely to happen *in vivo*, they hypothesised that glycine-induced-LTP may occur at distinct synapses, causing long-lasting enhancements of transmission in some select cases. At any rate, all of these studies demonstrate that synapses have several mechanisms at their disposal in addition to activity by which to regulate their morphology and efficacy. Thus, it may be that different parts of the nervous system use activity dependent and independent mechanisms in different ways at different times to achieve a final specificity of synaptic connections.

6.3 Activity Does Not Necessarily Induce Synaptic Modifications In Predictable Ways

One theory of synaptic plasticity involves the idea that synapses can respond to incoming information by detecting interactions between pre- and postsynaptic activity and register these associations as changes in synaptic weights (Frey &

¹ At least, independent of electrical activity, or, in other words, the activation of the post-synaptic membrane by presynaptic transmitter release.

Morris, 1997). However, attempts to investigate the nature of these changes have proved to be complex because manipulating pre- and postsynaptic activity in consistent ways does not always produce consistent effects at the synapses of interest. In particular, there are many examples in the hippocampus in which same stimulating regime has variable effects on synaptic strength. One of the earliest is a study by Coan et al. (1989), who found that LTP could not be produced using a standard induction protocol when hippocampal slices had been bathed in a low magnesium-containing medium. This effect was considered paradoxical at the time because the expectation was that removing magnesium from the extracellular solution should promote LTP by increasing the conductance of calcium in the postsynaptic membrane. Instead, it appeared that the threshold level of activity produced by the low-magnesium actually inhibited subsequent LTP induction. In an extension of this study, Huang et al. (1992) found that stimulating an input pathway with a tetanus that otherwise produced LTP, actually produced a depression of the synaptic response if the pathway had previously been stimulated at a low frequency. Interestingly, the prior stimulation regime on its own had produced no long lasting effects on the strength of transmission.

These studies suggest that the effects of activity on synaptic function may need to be evaluated in the context of the history of a synapse's activation. Recently, this idea has led to the proposal that the induction of LTP is associated with the setting of a "synaptic tag" at previously activated synapses, whose role is to sequester plasticity-related proteins (Frey & Morris, 1997). These proteins – as yet unidentified – stabilise transient synaptic changes, thereby extending their persistence. The

synaptic tag hypothesis strikes a chord with the results presented in **Chapter 4**, which showed that the resumption of activity following paralysis did not necessarily lead to synapse elimination. It is possible that the prior synaptic activation of the convergent inputs – perhaps before the onset of paralysis – left an enduring trace that affected the subsequent initiation or maintenance of the synapse elimination cascade.

6.4 Conclusions

Activity should perhaps begin to be viewed as only one of a number of factors that produce synaptic modifications. Consistent with the results presented in this dissertation, many different mechanisms probably contribute to neuromuscular plasticity. Indeed, there are likely to be many mechanisms that overlap between producing elimination and persistence of axonal inputs; for example, the activation of the AchR. Any biochemical processes set in motion by neural activity could conceivably play a role in synapse elimination, persistence, both of them, or neither, and the challenge now is to distinguish between these possibilities. On the other hand, recognition of the subtlety of activity might yield new interpretations of old data and provide new insights into one of the key questions in neurobiology: how is information stored in the nervous system?

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